Aspects of quality assurance in processing natural sausage casings

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Aspects of quality assurance in processing natural sausage casings

Aspecten van de kwaliteitsbewaking bij de productie van natuurdarmen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 8 januari 2009 des middags te 2.30 uur

door

Joris Jan Wijnker

geboren op 1 oktober 1967 te Aarle-Rixtel

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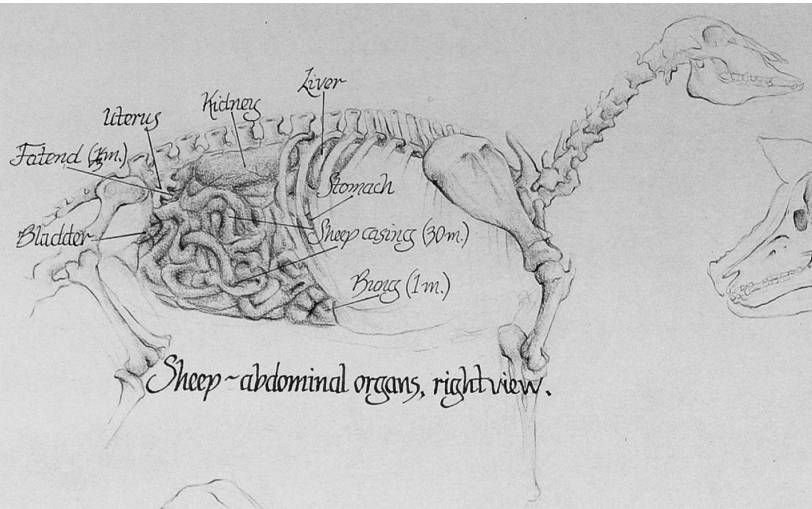
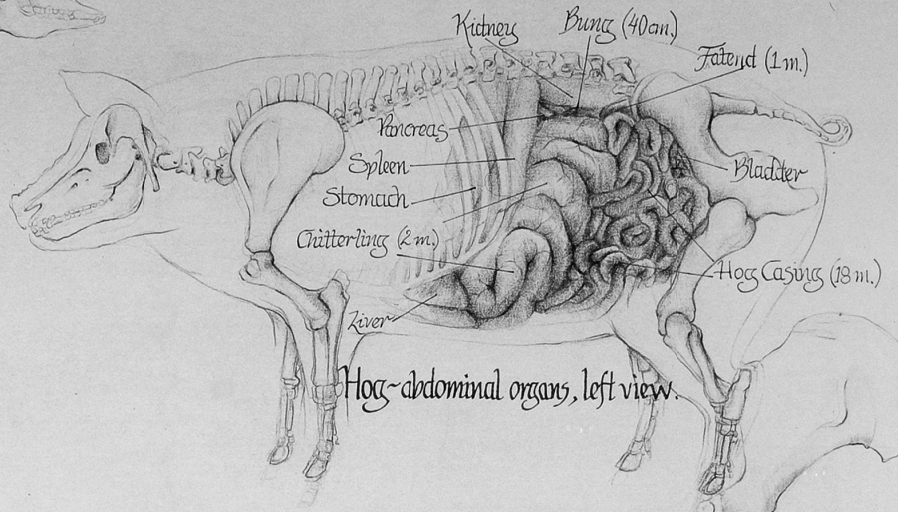
To Pieter and Marijke,

Brigitte, Max and Guus

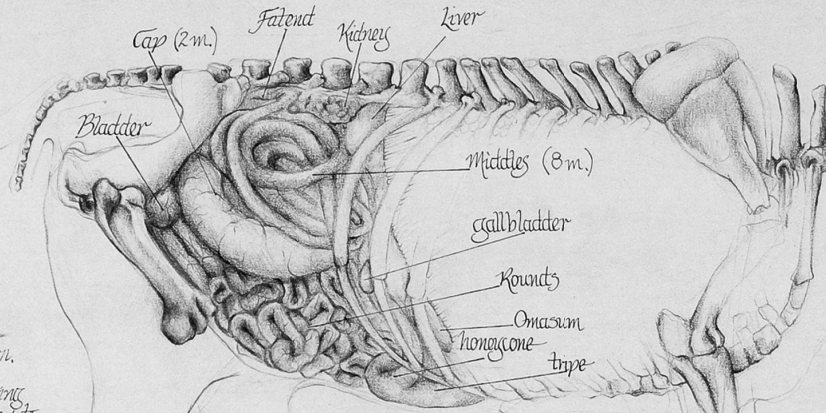
CONTENTS



Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 1

General introduction

Natural sausage casings ("casings") are traditional products that have been used in the production of meat specialities for centuries, and have remained virtually unchanged in function and appearance. A large variety of sausage is produced world-wide using the processed intestines of pigs, sheep, goats and cattle (and sometimes horses).
It is often assumed that sausages were invented by the Sumerians in the region that is Iraq today, around 4000 BC. Reference to a cooked meat product stuffed in a goat stomach like a sausage was known in Babylon and described as a recipe in the world's oldest cooking book 3750 years ago (Yale Babylonian collection, New Haven Connecticut, USA).

The Chinese sausage Làcháng, which consists of goat and lamb meat, was first mentioned in 589 BC. The Greek poet Homer mentioned a kind of blood sausage in his Odyssey (book 20, poem 25); Epicharmus (ca. 550 BC - ca. 460 BC) wrote a comedy entitled *The Sausage*. Numerous books report that sausages were already popular among the ancient Greeks and Romans.
During the reign of the Roman emperor Nero, sausages were associated with the Lupercalia festival. The early Catholic Church outlawed the Lupercalia Festival and declared the consumption of sausages to be a sin. For this reason, the Roman emperor Constantine banned the consumption of sausages. Early in the 10th century, the Byzantine emperor Leo VI forbade the production of blood sausages following cases of food poisoning, known in Germany as sausage poisoning.
Interestingly, the word sausage is derived from old French word *saussiche*, which could be found in a dialect spoken between 1000 and 1300 AD in a geographic region spanning the north of France and parts of Belgium and Switzerland. *Saussiche* comes from the Latin word *salsus*, meaning salted and creating a clear link to the long-known preservation method of casings using salt (www.wikipedia.org).

The art of producing sausages using animal intestines survived the fall of the Roman Empire and continued through the Middle Ages. With the development of cities throughout Europe, the butcher profession re-emerged, garnering great respect and even power. At the beginning of the 12th century, during the time of German Kaiser Heinrich V, butchers were recognized as eminent citizens.
Advances in meat processing were widely observed and sausage making was practiced throughout the Old World. As early as the 12th century, slaughterhouses in England separated more perishable materials (tripe, intestine) from carcass meat, and the French and Germans had set inspection requirements for meat products in the 13th century.
By the late medieval period, sheep were perhaps the most important domesticated animals; both individual farmers and monasteries owned huge flocks. However, the supply of casings could not meet the steadily growing demand of the sausage makers. Because of this, salt preserved casings became an important trade commodity across south and central Europe.
Intestines were praised as delicacies in medieval Europe. The German poet Kunig von dem Otenwalt in his song "Von der Küewe" (Küewe = cow) regarded large intestines as popular food and Steinmar (around 1200) spoke in his "Schmauslied" (Feast poem) about intestines ("Dermel") as pleasing and luxurious products. Later, around 1300 Johannes Hadlaub, a lyric poet, described that the German people highly valued meat products, including mesentery, intestines, viscera and sausages. In his painting "The Butcher Shop", the Dutch

painter Pieter Aertszen (1507 - 1573) depicted the details of common sausages amongst other meat specialities of that time such as ring sausages, link sausages and double links of small sausages.

The Butcher Shop (Pieter Aertszen)

In 1662, under the pseudonym Marcus Knackwurst, a book was written describing a number of famous sausage formulations with the emphasis on the use of natural sausage casings.
The late 18th and first half of the 19th century were the years of the industrial revolution. Improvements in meat preservation and processing methods resulted not only in dehydrated foods and dried meats, but also in new sausage production processes, again making use of animal casings as a natural envelop for meat preparations.

The public health and hygienic problems have for long been regarded as critical points in the practical application of casings. As a result the casings imported into Germany from foreign countries have been subjected to veterinary inspection since the introduction of the Meat Inspection Act ("Fleischbeschaugesetz") on June 3rd 1900. Problems related to the quality of casings, such as intestinal parasites and tuberculous knots as well as other sanitary defects, were discussed in the literature of that time (i.e. Von Ostertag, 1905). Gröning (1905) described the results of veterinary-sanitary examination of imported casings. In his studies, published in 1910 and 1920, he discussed the issue of hygiene and quality of imported casings (hog fat ends), including microbial red discolorations of salted products, commonly known as *"Red Dog"*. The problems related to the cleaning of casings concerned meat hygienists of that time. Schilling (1901) found in hog and beef casings several grams of faecal residue, consisting partly of straw

fragments, corn and animal hairs. Improvements in cleaning efficacy were permanently required.

During the first half of the 20th century the processing techniques of intestines into finished sausage casings were gradually improving. Knowledge of casing production was growing and new technology emerged. Von Ostertag (1905) described the methods of quality control of casings. New methods and machines were developed for the de-sliming and cleaning of casings (Heiss-Straubig, 1902; Nägele's cleaning machine – Patents Stohrer, 1919 and 1927).
Regulations, strictly applied to the growing import of casings, had an active and positive influence on the hygiene and technology of sausage preparation, not only in Europe but also around the world. The import of casings in Europe started to be more and more rigorously and successfully controlled, not only because of microbial risks but also because of the possible use of inedible salt and other inedible preservatives and additives.
Development of meat processing machines was an important stimulus for an accelerated growth of sausage production in this period. As a result, the need for casings increased which encouraged both further improvement of processing and preserving methods of casings and paved the way for the invention of new alternative types of casings from natural and man-made materials (Savic and Savic, 2002).
This change from a traditional style of sausage production to a uniform industrial approach did not make natural casings obsolete. Dr. Gisela Panzer wrote in 1977 that sausages stuffed in natural casings are, due to their non-uniform appearance, clearly distinguishable from mass-produced products and are therefore acceptable as a higher quality product. Prof. Sakata (1998) stated that natural casings achieve marked consumer preferences over artificial casings due to their better bite-resistance (The -"knack"- in knackwurst).
A quick search on the internet shows that sausages have not lost their appeal to the modern consumer. A country well-known for its sausage tradition is Germany, where the average consumer buys some 30 kilos of sausage per year – representing half of his annual meat consumption. This high consumption is associated with a range of more than 1,500 different kinds of sausage, produced mostly locally and with regional variations regarding composition, smoking and spicing techniques (Wijnker, 2006).

Despite the appreciation of sausages as meat products, the attention of the scientific community in this product remained limited. Apart from a handful of studies, most investigations focussed on meat (products) addressing hygienic issues and the control of contagious animal diseases. However, since the 1970s natural casings have been studied more closely and these investigations are presented in more detail in the introduction sections of the following individual chapters.

Production of natural sausage casings

Anatomy

From pigs the entire intestinal tract is used for the production of casings, specifically the small intestines (duodenum, jejunum, ileum), bung (caecum), large intestines (colon ascendens & transversum), after end (colon descendens) and fat end (rectum). From sheep, only the small intestines are used, particularly the duodenum and jejunum and sometimes also the ileum. The intestinal tract of cattle is also used entirely with the exception of the ileum. Its shape differs too much from the jejunum to produce the classic beef rounds and is therefore removed prior to the cleaning process and destroyed. Beef casings are produced from the weasand (oesophagus), small intestines (duodenum, jejunum) which are processed into beef rounds, bung (caecum), large intestines (colon) which are processed into beef middles, and bladders (Ockerman and Hansen, 2000).

Although there is quite a large variety in shapes and sizes of the intestinal tract between the different species used for the casing production, their basic anatomy and function are remarkably similar. The intestinal wall is composed of four basic layers (Figure 1).

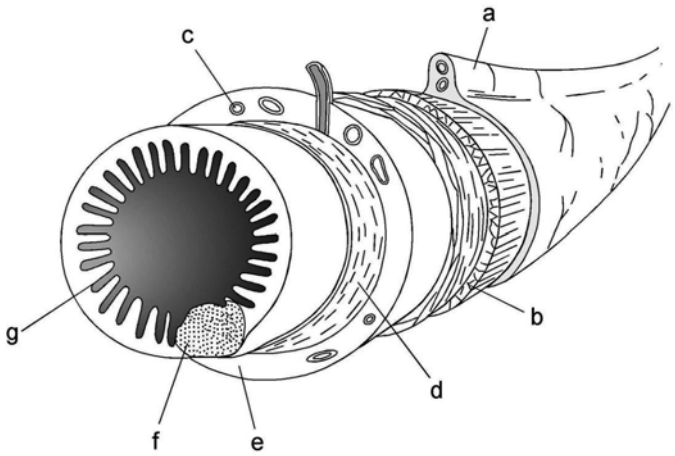


Figure 1: Schematic diagram of sheep small intestine showing mesentery and serosa (a), inner and outer muscle layers (b), submucosal blood vessels (c), muscularis mucosae (d), submucosa (e), lymphoid nodule (Peyer's patch) (f) and tunica mucosa (villus and crypt layers) (g). The tunica mucosa, the muscularis, the serosa and Peyer's patches are removed during processing, so the natural casing consists of only the submucosa (e).

The tunica serosa is the outermost layer covering the intestinal tract. The tunica muscularis consists of two layers of smooth muscle, with an inner layer in a circular and an outer layer in a longitudinal orientation. The tunica submucosa, lying beneath the tunica muscularis, has a microstructure characterized by a network of collagen fibres (type I), elastin and blood vessels of different sizes. For hog and sheep casings, this submucosa is the remaining layer of the intestine after processing and forms the natural sausage casing (Figure 2).

Figure 2: Fully processed sheep casing

The tunica mucosa is the innermost layer of the intestinal tract and lines the lumen. Embedded in the mucosa lies lymphatic tissue which occurs irregularly along the length of the small intestine as isolated lymphoid nodules (lymphonoduli solitarii), but tend to be most prominent in the ileum. These aggregated lymphoid nodules (lymphonoduli aggregati) are known as Peyer's Patches and are anatomically located on the convex side of the intestine opposite to the mesenteric attachment.

Taking a sheep casing as example, figure 3 shows the full thickness of the uncleaned small intestine and figure 4 shows the tunica submucosa as remaining tissue layer after the cleaning process is finished. A cleaned sheep casing is on average 0.11 mm thick, whereas a cleaned hog casing, also comprising only of the submucosa, is 0.32 mm (Bartenschlager-Blässing 1979; Koolmees and Houben 1997).

Figure 3 & 4: Sheep casing, before and after cleaning (HE colouring, magnification X 25)

A study done by Nishiumi and Sakata (1999) on the histological and biochemical properties of casings found that each casing was composed of numerous sheet-like layers of collagen fibres in a criss-cross arrangement. Differences in gross organisation of the collagen fibres was not observed, but the outer layers of Chinese sheep casings contained more stretched collagen fibres and was packed more densely than Australian sheep casings. These casings had relative fine collagen fibres of loosely packed fibrils. Casings contained approximately 2 % elastin accumulated in blood vessels, without any differences in morphology, localisation and density between samples of different species and origin. Only Japanese hog casings seem to be an exception with lower elastin content. According to Nishiumi and Sakata, the mechanical properties are determined by the size, arrangement of collagen fibres and heat-solubility of collagen present in casings. Differences in the heat-solubility of collagen in casings of various origins may therefore influence usability and palatability of these casings and subsequent studies to tenderize casings will be discussed below.

Processing

The subsequent cleaning process of small intestines can vary between species and geographical location of the cleaning operation. Clear differences exist in how the intestines are pulled from the viscera, being either with a knife, by hand or by machine (Smits and Keizer, 2003). Contrary to hog casings, sheep casings are fermented prior to the cleaning process. After the manure is stripped from the intestines, sheep casings are placed overnight in a cooled storage during which the mucosa will degrade for easier removal. In general for hog and sheep casings, the manure is stripped out of the small intestines, the mucosa is crushed and removed in various steps and the outer layers, being the tunica

muscularis and tunica serosa, are scraped off, leaving the tunica submucosa (Fisher and Schweflinghaus, 1988; Ockerman and Hansen, 2000).
The porcine large intestines are mostly processed into hog chitterlings and fat ends by hand. All layers remain identifiable, whereas it depends on the operation if the mucosa is (partially) removed (Schweigmann and Seeger, 1988; Ockerman and Hansen, 2000).
In contrast to the extensive processing of hog and sheep small intestines into casings, beef casings retain all original layers after cleaning (Botka-Petrak et al., 2001). A pilot study on the histology of beef casings (Koolmees, 1998) indicated that although most of the tunica mucosa is removed from the small intestines, the tunica muscularis and serosa can be clearly identified and that Peyer's Patches also remain present.
In order to determine whether a difference existed in cleaning efficacy between manual and mechanical processing techniques, a comparative histological study was done by Koolmees et al. (2004) using sheep casings. Results showed that no significant differences existed between both techniques and that no lymphatic tissue (Peyer's Patches) remained after cleaning.

Mechanical and biochemical properties of casings

An important issue in the quality of casings is their usability for the stuffing process of a great variety of sausages and the consumers' preferences regarding texture and tenderness. Although these technological aspects are beyond the scope of this thesis, a brief overview is warranted to indicate certain developments.
A study (den Reijer, 1996) done on the toughness of casings in the production of smoked sausages, indicated that many factors not directly related to the casings themselves have a significant influence on the toughness perception. Higher fat content of the sausage meat batter and improved smoking procedures enhance the overall quality.
Various additives, used during the initial curing process of the casings (Bakker et al., 1999) prior to storage in salt, were tested for their influence on the mechanical properties of casings. Combinations of citric acid / Na_3-citrate, lactic acid / Na-lactate or phosphates (Na_3PO_4 / Na_2HPO_4) and NaCl improved the mechanical properties, with prevalence for phosphates on the final usability of the casings (Verkleij and Keizer, 2003).
Japanese studies aiming to tenderize hog casings evaluated the effect of lactic acid and pepsin (Sakata et al., 1998) or high pressure treatment in combination with lactic, acetic or citric acid (Nishiumi et al., 2005), whereas Reichert (1996) studied the effect of a proteolytic fruit enzyme solution on casings after stuffing. A recent study (Nakae et al., 2008) on the influence of trisodium phosphate (TSP) gave a clearer indication than Bakker et al. (1999) that phosphates can reduce the maximum force and breaking strain of casings.
An overall conclusion could be that phosphates are preferred over organic acids and other substances, although a comprehensive study would be required to make a full assessment of the different treatments and their effects on various quality parameters of casings. Such an attempt was made by Schwanz and Schnäckel (2007a; 2007b) who developed an elaborate set-up to test various additives and their influence on the quality of casings. Interestingly, their results also showed that soaking desalinated casings in plain water (45 °C) for around 30 minutes prior to stuffing was sufficient to create an acceptable bite.

Microbial contamination of casings

Bacteria

As casings are cleaned in batches at water temperatures around 40 °C, microbiological cross-contamination and bacterial outgrowth is evident. Koolmees and Houben (1997) illustrated the bacterial load of casings at various cleaning steps, underlining the necessity to take appropriate actions to prevent quality loss and possible food safety risks.

	Hog casings (counts cfu / g)	Sheep casings (counts cfu / g)
Total aerobic count	$10^{5.8}$	$10^{6.5}$
Enterobacteriaceae	$10^{4.4}$	$10^{4.3}$
S. aureus	$10^{3.4}$	$10^{4.3}$
Salt tolerant org.	$10^{3.5}$	$10^{3.4}$
Lactic acid bacteria	$10^{4.7}$	$10^{5.5}$
Bacterial spores[a]	$< 10^{2}$	$< 10^{3}$

a) if detected

Table 1: Bacteriological results of freshly cleaned hog and sheep casings

Riha and Solberg (1970) investigated the microflora of salted sheep casings used for the production of fresh pork sausages. Various bacterial contaminants were identified including *Bacillus, Pseudomonas, Clostridium, Micrococcus, Proteus* and *Lactobacillus*. Casings were stored in either dry-salt or saturated brine and sampled within 4 weeks after production. Aerobic total plate counts were comparable between the different samples and corresponded to the counts found immediately after cleaning (see table 1). However, Riha and Solberg (1970) did not mention the exact period between casing production and sampling. They acknowledged the wide variety of spore-forming bacteria present and concluded that the potential shelf-life reduction of fresh sausage contributed by the casing material is minimal, provided the sausage is processed properly and stored at 4 °C.

In 1974 the study by Gabis and Silliker was published on *Salmonella* in beef, sheep and hog casings. Results indicated a complete removal of *Salmonella* ($10^{6.2}$ cfu / g) in dry-salted beef and sheep casings after 7 days (stored at 6 °C) or after 21 days in hog casings stored in saturated brine (stored at 6 °C). A study conducted by Bartenschlager-Bläßing (1979) reported the absence of high bacterial counts in sheep and hog casings and a survey done by Houben (2005) on the presence of *Salmonella*, *Listeria* and *Clostridum* spores in dry-salted casings indicated that only spores were present after prolonged storage. In addition, Schweigmann and Seeger (1988) showed that casings produced from large intestines are preserved in less salt than small intestine casings and can therefore contain higher microbial counts.

Bockemühl (2000) studied the presence and survivability of certain pathogens using fresh and salted beef, sheep and hog casings. *Listeria monocytogenes* was confirmed in beef casings and *L. monocytogenes*, *Salmonella* and *Campylobacter* were confirmed in fresh hog casings after 30 days of storage in dry-salt at 15 °C. These bacteria were no longer present except for *Salmonella* and *L. monocytogenes* in hog casings. However, information on salt concentrations or actual bacterial counts was not given in this report, preventing any clear conclusions.

A comprehensive study by Wirth (1990, 1994) reported not only the microbial aspects of casings after prolonged storage but evaluated also whether or not the casings were still usable for further processing. Storage at 22 °C provided lower bacterial counts than 4 °C, although the quality of casings was better preserved at lower temperatures. Wirth concluded that most likely the influence of light and higher oxygen concentrations were detrimental to the quality loss, a situation specific for this experiment and in contrast to the general storage in closed casks.
All these results indicated that the high bacterial counts found during and directly after the cleaning procedure could be countered by correct curing and subsequent storage in dry-salt or brine. However, the specific concentration-dependent influence of salt and brine on various bacteria has not been clearly determined and remain to be studied.

Prions

A recently emerging sanitary problem is the possible presence of infective prions in tissues of slaughter animals, particularly in tissues derived from cattle. Normal prion protein (PrP^C) is a glycoprotein expressed by neurons and other cells and functions as a copper-dependent antioxidant (Brown et al., 1999). The transition of healthy, routinely synthesized PrP^C to infectious prion proteins (PrP^{Sc}), based on the prion dimer theory of Prusiner, follows the merging of a normal (healthy) and an infectious prion molecule to form a PrP^C–PrP^{Sc} heterodimer, in which the normal PrP^C molecule is restructured into PrP^{Sc} (Prusiner, 1998).
All known forms of Transmissible Spongiform Encephalopathies (TSEs) are characterized by the extracellular aggregation of infectious prion proteins that can cause neurodegenerative disease within the CNS by forming plaques known as amyloids, which disrupt the normal tissue structure (Doherr, 2007). This disruption is characterized by "holes" in the tissue with resultant spongy architecture due to the vacuole formation in the neurons. These progressive lesions cause impairment of brain function (memory, behaviour, movement) and most often result in death of the patient. Known prion diseases are Bovine Spongiform Encephalopathy (BSE) and Variant Creutzfeld-Jakob disease (vCJD) in humans, which has been associated with the exposure to BSE prions via animal products.
As a result of the initial epidemiological assessment in the 1980ies and in consideration of the possible link between BSE and vCJD, restrictive measures were put in place. This resulted by the end of 1997 in the listing of all cattle tissue types and organs that could be contaminated with BSE as Specified Risk Material (SRM), including brain, spinal cord, vertebral column and the entire intestinal tract from duodenum to rectum of cattle and the ileum of sheep.
The plausible route of infection via contaminated feed suggested that the intestinal tract of cattle could play a major role in the uptake of the BSE agent. From the first studies done at the European reference laboratory for BSE in the UK it became apparent that only the distal ileum, containing higher quantities of lymphatic tissue than the jejunum or duodenum, was found to be infective (Wells, 1994). Subsequent studies did not find any other part of the intestines to be positive for BSE prions (Terry, 2003; Buschmann and Groschup, 2005; Hoffmann et al., 2007).
To determine the actual BSE infectivity risk in cattle intestines more studies are required to generate sufficient data for statistical analysis. However, the options to perform such studies are extremely limited and a distinction needs to be made between uncleaned cattle intestines and processed beef casings regarding the

possible presence of BSE-related infectivity. A different approach would be to determine the contribution of beef casings in a human exposure assessment. This route has been used for various beef products and has led to several amendments of the SRM list.

Viruses

As casings are sourced, processed and subsequently shipped to sausage producers worldwide, they have been identified as possible carriers of infectious animal diseases, such as food-and-mouth disease (FMD) and classical swine fever (CSF). Overviews given by Blackwell (1984) and Farez and Morley (1997) provide limited information on casings and when reviewing the original studies, casings were never the intended subject of the study.

McKercher et al. (1978; 1980) mentioned that residual infective FMD virus remains in untreated processed natural casings for as long as 250 days. Unfortunately the authors provide neither reference to the original studies on the natural casings involved, nor information on processing and storage conditions of these casings (temperature, pH, salting).

Panina et al. (1989, 1992) showed that lactic acid formation in fermented sausages led to complete loss of FMD virus infectivity and that CSF virus could survive for at least 75 days in cured sausages. However, the casings used for the production of these sausages did not originate from infected animals.

Only two studies were found which actually investigated CSFV infectivity in processed casings (Helwig and Keast, 1966; McKercher et al., 1980). However, information from these studies was either incomplete or based on incomparable processing methods to allow for an accurate risk assessment.

Böhm and Krebs (1974) were the first to report on different FMD virus titres in specific tissues of experimentally infected sheep including intestines and freshly cleaned casings. They also confirmed the efficacy of a 5-minute treatment with 0.5 % citric acid on infected sheep casings to inactivate the FMD virus. Although this was also mentioned by McKercher et al. in 1978, no other studies on FMD virus inactivation in natural casings are known.

As a result from the absence of specific data for FMD and CSF virus survival in processed casings, risk assessments for the international trade in casings have been extrapolated from other products, with either insufficient risk reduction or resulting in unnecessary trade restrictions. Therefore it will be of great relevance to clarify this situation and to determine the potential threat of casings in the dissemination of these contagious animal diseases.

Cross-contamination of casings via salt

Halophilic bacteria are introduced to casings not via the original contamination of the uncleaned animal intestines or cleaning process but via the salt used as preservation agent. The salt used for preservation must meet the “Food Grade” requirements included in the Codex Alimentarius (Codex Stan 150-1985 1) but can be of different origin and produced under various conditions. Solar evaporation techniques are used, salt is mined or pumped as brine from underground salt layers and processed yielding evaporated salt of the highest purity. Although the presence of halophilic bacteria has little relevance in respect to food safety, they play an important role in product quality due to odour development, discoloration and proteolysis of the casing due to bacterial outgrowth.

Known halophilic bacteria to cause “red dog” (Rust, 1988) on the surface of casings are *Halobacterium salinarum* and *H. cutirubrum* and require a salt concentration of more than 150 g NaCl per litre water. These bacteria are gram negative facultative aerobic, non-spore forming rods or cocci, depending on growth conditions (Labots, 1967; DasSarma, 2001). They produce a red-orange carotenoid pigment for self-protection against the high levels of ultraviolet radiation in their normal habitat (salt lakes, solar evaporation ponds) and can also produce buoyant gas vesicles. Its purpose is to enable these bacteria, whose primary metabolism is aerobic, to float to the more oxygenated surface areas (DasSarma, 2001).
Studies done by Labots and Krol (1964) indicated that increased growth occurs at salt concentrations higher than 20 % (wt/wt), temperatures higher than 20 °C and neutral pH. Removal of halophilic bacteria was suggested by flushing casings in potable water (> 30 minutes) and storage at temperatures around 10 °C to prevent outgrowth of any remaining bacteria.
Wirth (1990, 1994) reported on the microbial contamination of casing samples from various origins after prolonged storage (3, 6 & 12 months) at 4 or 22 °C. Results showed only a clear reduction in halophilic bacteria after prolonged storage for 12 months at either storage temperature. Results also showed that the origin of the casings and therefore the origin of the salt played a major role in the original contamination with halophilic bacteria.
Bakker et al. (1999) used freshly cleaned casings in their experiments to determine the effects of initial curing with additives on microbial quality and mechanical properties. Salt of unknown origin was used and halophilic bacteria and presence of “red dog” were confirmed in slush cured and dry cured sheep casings stored at different temperatures for 3 and 6 months. It was shown that, whether curing additives (citrate, lactate or phosphate) were used or not, spoilage occurred by halophilic bacteria with the distinctive red discoloration and smell occurring after 3 months storage at 20-40 ºC and after 6 months also at 10 ºC in slush cured sheep casings.
These results confirm the original findings by Wirth (1990, 1994) that halophilic bacteria can remain present after prolonged storage. However, they also contradict the results found by Labots and Krol (1964) that a lower storage temperature prevents further outgrow. Most likely a combination of original contamination, storage time and temperature determine whether halophilic remain present. As a result the focus should lie on prevention in stead of elimination. Adequate control of salt quality and prevention of cross-contamination of contaminated batches should further reduce the loss of quality due to halophilic bacteria.

Recent developments in the preservation of casings

Several studies have been done recently on the usability of gamma irradiation as preservation method for casings (Trigo and Fraqueza, 1998; Byun et al., 2001; Jo et al., 2002), culminating in the study by Chawla et al. (2006) on the inclusion of gamma irradiation into the principle of Hurdle Technology.
Trigo and Fraqueza (1998) used fresh, locally produced, hog casings and dried beef casings. Analysis of the bacterial population prior to irradiation yielded high counts (i.e. total aerobic count 7.54 $\log_{10}$ cfu / g casing), which could only be fully eliminated in the fresh hog casings after exposure to 10 kGy of gamma radiation.

The studies by Byun et al. and Jo et al. (2001; 2002, one experimental set-up, two separate articles), used freshly salted and semidried hog and sheep (lamb) casings. Casings were exposed to gamma radiation either as salted product or after washing in de-ionized distilled water, used for sausage production (Bratella Weiss Wurst) and subsequently a bacterial and sensory evaluation was done. A combination of washing and exposure to 5 kGy was sufficient to eliminate all Enterococci and coliform bacteria, although other aerobic bacteria remained present. Sausages produced using irradiated casings had an increased tenderness at both 3 and 5 kGy but there were no significant differences found in the sensory analysis in comparison to non-irradiated sausages (flavour, colour, texture).
The final study by Chawla et al. (2006) used fresh, locally produced, sheep (lamb) casings, salted with NaCl (10 % w/w) to reduce water activity from 0.95 to 0.80, which were irradiated at 5 and 10 kGy and stored at ambient temperatures. Microbiological analysis was done up to 90 days post irradiation and sensory analyses of sausages produced with these casings were done at day 0 and day 30 post irradiation. Only the highest dose of 10 kGy succeeded in eliminating the total aerobic count ($>10^6$ cfu / g casing) and spores of sulphite reducing *Clostridia* (10^3 cfu / g casing). The sensory evaluation (colour, odour, texture) revealed no significant differences at either interval or radiation dose. Contrary to Byun et al. (2001), no textural changes indicating an increased tenderness were observed.
Based on these studies a combination of reduced water activity and radiation processing can improve the safety of natural casings without affecting their functional properties. However, casings are generally stored for a prolonged time in either dry salt or saturated brine prior to sausage production. For instance, a study done by Gabis and Silliker (1974) showed that after 3 weeks of storage in dry salt at 6 °C, no *Salmonella* could be found. Secondly, Houben (2005) found only sulphite reducing *Clostridia* spores in dry-salted hog and sheep casings. These studies may question the necessity to use gamma irradiation as another hurdle whereas preservation in salt after a certain storage period is sufficient to remove microbial contamination of casings, with bacterial spores as the known exception.

Ozonated water was used in a study by Benli et al. (2008) to determine its efficacy on the preservation of hog casings, taking into account its effects on the biomechanical properties after treatment. Ozone is a strong oxidant and ozonated water has been reported to effectively kill spoilage micro-organisms, environmental and faecal contaminants and food-borne pathogens in low ozone demand media. However, a high protein environment negatively affects the stability of dissolved ozone. Results indicated that prolonged exposure of casings to ozone, necessary to obtain a relevant reduction of resident micro-organisms, would lead to a substantial weakening of the casing. Subsequently the use of this technique for the preservation of casings was rejected.

Scope and aim of the thesis

The most common method used for the preservation of casings remains salting, either with dry-salt or with saturated brine, allowing the production of a high quality product with a long shelf-life without strict storage and transport requirements.
In order to further substantiate this traditional technique, but also to expand on its abilities and augment its efficacy, several studies are included in this thesis on a variety of subjects. **Chapter 2** focuses on the bactericidal effect of salt, either as dry-salt or as brine in various concentrations. The aim was to determine which brine concentration is minimally required to reach an acceptable bacterial contamination level and whether recommendations can be made on a minimum storage period to reach this level. The results further substantiate the quality and efficacy of salt preservation of casings.
Spore-forming bacteria can survive most preservation techniques used for casings because of their ability to form spores when the vegetative state of the bacteria is being threatened. Since *Clostridia* have been identified as a possible quality risk (food spoilage, health risk), **Chapter 3** describes the effects of nisin on the outgrowth of *Clostridium sporogenes* spores in a new model developed for casings. Sterile, gamma-radiated casings were tested and the inhibitory effect and biological availability of nisin was determined. The outcome may assist in a further reduction of Clostridia contamination in casings.
Previous studies on the pathogenesis of BSE had shown that only the ileum seems to be an infectious part of the cattle intestines. As the ileum can be clearly identified and therefore removed prior to the cleaning process, the remaining small intestines might therefore remain acceptable for human consumption. **Chapter 4** presents a quantitative histological analysis of bovine small intestines before and after processing into beef casings. The study focuses on the removal of BSE-related tissues (neural and lymphatic tissue) during the normal cleaning process and presents a calculation of the amounts to which the consumer is exposed per portion of sausage. These results can be included in a quantitative BSE risk assessment in order to better understand the role of beef casings in BSE exposure.
Both foot-and-mouth disease and classical swine fever have been identified as high-risk animal diseases with a major impact on animal welfare and economic repercussions. **Chapters 5 and 6** describe the studies done on the inactivation of FMD and CSF virus in cleaned casings, either by salt or by salt mixed with certain phosphates. The aim of these studies was not only to determine the efficacy of the various treatments but also to assess whether or not recommendations can be made regarding storage requirements to remove any infectivity.
In order to allow the determination whether or not casings were treated with phosphate supplemented salt, an existing phosphate analysis method was evaluated and validated for the application in the testing of casings. **Chapter 7** describes this study, with the aim to present a standard method to determine whether or not casings have been treated against FMD and CSF infectivity.
Finally, in **Chapter 8** the findings of these studies are summarised and discussed, resulting in an understanding of several quality aspects of casings and in the identification of specific areas for future research.

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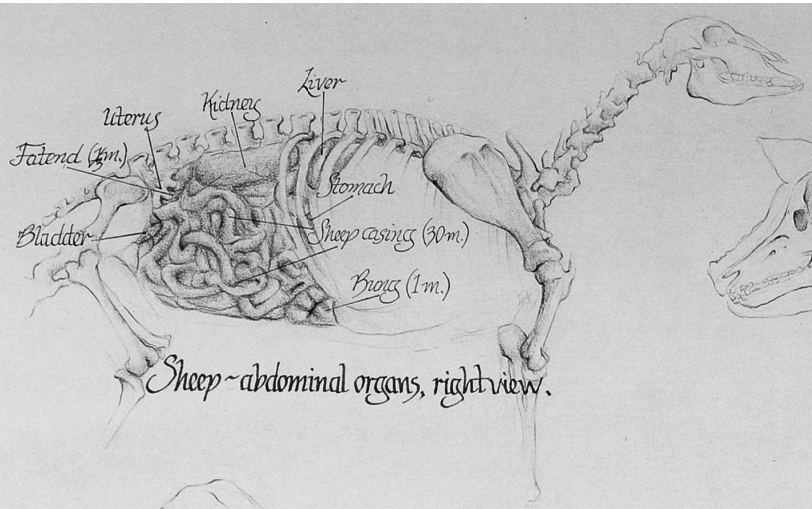
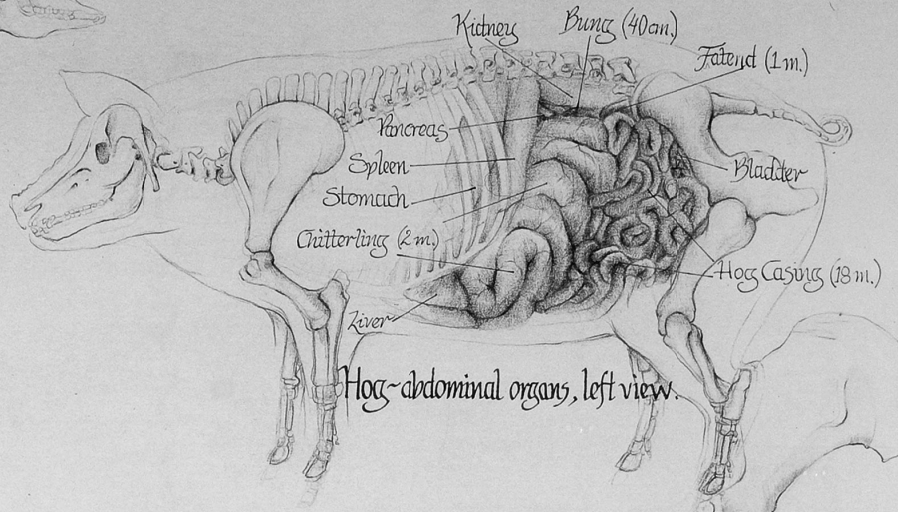
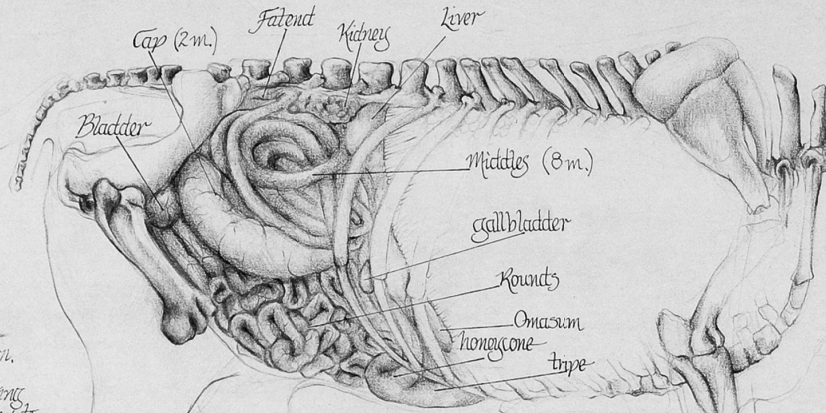


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 2

Antimicrobial properties of salt (NaCl) used for the preservation of natural sausage casings

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Abstract

The antimicrobial properties of salt (NaCl) used for the preservation of natural sausage casings were studied by investigating the survival of six bacterial species in casings at different water activity (a_w) levels. Individual sheep casings were inoculated with circa 10^5 colony-forming units (cfu) g^{-1} of *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes, Staphylococcus aureus*, *Clostridium perfringens* and 10^2 cfu g^{-1} of *Escherichia coli O157:H7*.
The casings were stored at 20±1.5 °C in different brines and dry salt, giving a_w-levels of 0.90 a_w, 0.87 a_w, 0.85 a_w, 0.83 a_w en 0.75 a_w. Samples were taken at day 1, 3, 6, 8, 13, 20, 27 and 30 after inoculation and the number of bacteria present was determined. Based on survival curves, death rates (day^{-1}) were calculated to quantify the reduction in $\log_{10}$ cfu g^{-1} per day.
The influence of a_w on death rates was higher for Gram-negative bacteria than for Gram-positive bacteria. The death rates were overall higher for Gram-negatives than for Gram-positives. No clear reduction in the survival of *C. perfringens* in relation to any a_w level was observed in this study.
These results indicate that the antimicrobial properties of salt used for the preservation of natural casings are sufficient to reduce the bacterial contamination (except for *Clostridium* spores) well below acceptable levels at a water activity level of 0.85 or lower during a 30-day storage period.

Introduction

As sausage is known to be the oldest and longest established form of processed meat, the intestines of sheep, hogs and cattle have been used for thousands of years as an edible container for this well-regarded product. The basic technique for the cleaning of casings has also remained unchanged over all this time (Ockerman and Hansen, 2000) and a comparative study (Koolmees et al., 2004) revealed no significant difference in the cleaning efficacy of manual or mechanical cleaned sheep casings. In general, the remaining submucosa layer is known as the natural sausage casing (Bakker et al., 1999) and, based on the animal it originates from, is used for a wide variety of sausages (Houben, 2005).
Casings are usually preserved by salting, curing and/or drying (Fischer and Schweflinghaus, 1988). By using salt, the water activity (a_w) of the product is decreased and together with temperature and pH, is one of the major parameters influencing bacterial growth and survival.
The lethal effect of a low a_w (Gutierrez et al., 1995) is linked to the fact that the turgor pressure in a cell is established as a result of the intracellular a_w and a_w in the surrounding medium. A process known as plasmolysis (Csonka, 1989), describes how hyperosmotic shock causes an instantaneous efflux of water, accompanied by a decrease in the cytoplasmatic volume of the cell. A universal response to the temporary loss of turgor is the cytoplasmatic accumulation of so-called 'compatible solutes' (Sperber, 1982; Beals, 2004). Compatible solutes are small organic molecules which share a number of common properties: amongst others they are soluble to high concentration and can be accumulated to very high levels (up to 1M) in the cytoplasm of osmotically stressed cells; they do not alter enzyme activity and may even protect enzymes from denaturation by salts. Known compatible solutes are i.e. betaine, glycerol, sucrose and proline (Gutierrez et al., 1995). Whether this response to the lowering of a_w can prevent cell-death is complex and influenced by multiple factors, both intrinsic and extrinsic and differ within food types, processes and amongst types of flora involved (Lenovich, 1987).
A study done by Park and Beuchat (2000) on the survival of *Escherichia coli O157:H7* in potato starch clearly suggests that bacterial viability studies at reduced a_w are greatly affected by the composition of foods. For this reason only casings can serve as its own model to assess the viability of specific pathogens at reduced a_w.
Except for a single study on the survival of *Salmonella spp*. (Gabis and Silliker, 1974) and a survey on the presence of certain pathogens in dry-salted casings (Houben, 2005), the influence of a reduced a_w on the survival of bacteria in preserved casings (dry-salted or brine) has never been investigated. The object of this study was to examine the antimicrobial properties of salt based on water activity, made visible by the supposedly reduced survival of six relevant food pathogens added to casings preserved in dry salt and several different brine concentrations. Additionally, water activity was measured as a novel parameter with possible predictive qualities for the preservation in dry salt and brines.

Materials and methods

Since no microbiological criteria were described for casings in existing legislation, the European Natural Sausage Casings Association (ENSCA) agreed on certain microbiological recommendations for salted casings in 1996. These

recommendations were incorporated into the HACCP manual for the processing of natural sausage casings (Fischer and Krol, 1997, Table 1). The EU -CRAFT-Project (BRE 2.CT 94. 1495), commissioned by ENSCA illustrated the microbiological load of sheep and hog casings at different steps of the cleaning process (Utrecht University, 1995; Ockerman and Hansen, 2000), with 4.31 $\log_{10}$ cfu g^{-1} for S. aureus and 4.26 $\log_{10}$ cfu g^{-1} for Enterobacteriaceae after the final cleaning step. Since sheep casings were found to be more contaminated than hog casings, sheep casings were used for this study. The choice of relevant food pathogens was based on readily available information on the internet (www.food-info.net, www.cfsan.fda.gov).

Species	cfu[a] / g acceptable	cfu / g maximum limit
Total aerobic count	$<10^5$	5.10^6
Enterobacteriaceae	$<10^2$	10^4
S. aureus	$<10^2$	10^3
Sulphite reducing *Clostridia* - spores	$<10^2$	10^3

a) colony forming unit

Table 1: European Natural Sausage Casings Association (ENSCA) microbiological recommendations

Bacteria

The following bacterial species were chosen: *Escherichia coli* (ATCC 10536), *Escherichia coli O157:H7* (ATCC 43895), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 7644), *Staphylococcus aureus* (ATCC 29213) and *Clostridium perfringens* (ATCC 13124). Each bacterial species was grown in 10 ml TSB (Tryptone Soy Broth, Oxoid CM 129,) for 24 hours at 37 °C, diluted and counted to create final inocula (approximately 10^5 cfu g^{-1}). An exception was made for *Escherichia coli O157:H7* where a lower final inoculum was made of 10^2 cfu ml^{-1} due to the high pathogenicity of this species. Specific media for culture and enrichment are listed in Table 2 for each bacterial species.

Species	Plating medium	Pre-enrichment	Enrichment
E. coli ISO 7251	MacConkey Agar (24h, 37°C)		Buffered Peptone Water (BPW) (24h, 37°C)
E. coli O157:H7 ISO 16654	CT SMAC (24h, 37°C)		mTSB + novobiocin (16-20h, 41.5°C)
S. typhimurium ISO 6579	Brilliant Green Agar (BGA) XLD agar (24h, 37°C)	Buffered Peptone Water (BPW) (24h, 37°C)	Rappaport-Vassidialis Soy peptone broth (RVS) (24-48h, 42°C) Selenite broth (24h, 37°C)
L. monocytogenes ISO 11290	Compass Medium (24h, 37°C)	half-Fräser broth (24h, 30°C)	Fräser broth (24h, 37°C)
S. aureus ISO 6888	Baird-Parker Medium (BP) (24h, 37°C)		Giolitti Cantoni broth (24h, 37°C)
C. perfringens ISO 7937	Iron Sulfite Agar (ISA) (24h, 37°C) anaerobic		Rapid Perfingens Medium (RPM) (48h, 37°C)

Table 2: ISO methods and media used for plating and enrichment

Sample preparation
Dry-salted Australian sheep casings (AA 22-24) were taken from stock (courtesy Van Hessen bv, the Netherlands) and were previously cleaned, scraped, salted and processed according to Company Standard Operating Procedures. The casings were desalinated in water, checked for holes and divided into pieces of approximately one meter in length. The casings were tied up at one end, re-salted and stored under ambient conditions according to the company's specifications.

Preservation media
To preserve casings under controlled conditions, 4 different brines with specific salt concentrations (molarity: 2.8, 4.0, 5.2 and 6.2 M) and dry salt were prepared for storage. Final a_w values were measured during storage of the inoculated casing samples (AW LAB Set H, Novasina, Switzerland). Temperature and pH measurements were made during the entire period of 30 days at regular intervals.

Inoculation procedure
The prepared casings were rinsed in brines with similar molarities as their final preservation brine to remove any salt particles. The cleaned casings were weighed and corresponding amounts of inocula in a 1:1 ratio with TSB were brought into the casings using a blunt-tipped needle resulting in a primary contamination of 4.68 $\log_{10}$ cfu g^{-1} for *E. coli*, 2.30 $\log_{10}$ cfu g^{-1} for *E. coli O157:H7*, 4.78 $\log_{10}$ cfu g^{-1} for *S. typhimurium*, 5.29 $\log_{10}$ cfu g^{-1} for *L. monocytogenes*, 4.51 $\log_{10}$ cfu g^{-1} for *S. aureus* and 4.18 $\log_{10}$ cfu g^{-1} for *C. perfringens*. The open end of each casing was tied up and the inocula were distributed through each casing. Subsequent batches of 8 pieces (corresponding in inoculum and preservation medium) were weighed into an equal volume of its specific preservation medium and shaken (120 oscillations min^{-1}) for one hour, after which they were returned to their original brine. All inoculations were done in duplicate and representative samples of the casings were checked prior to inoculation on the presence of contaminating bacteria in order to create a negative control; no contamination was present. The procedures used for these checks prior to inoculation were similar to the procedures used for the final microbiological analysis (Table 2).

Microbiological analysis
At 1, 3, 6, 8, 13, 20, 27 and 30 days after inoculation, a single piece of casing for each a_w was cut into 3 cm pieces. To avoid any negative interference from the salt, the casings were weighed into a 19-fold volume of buffered peptone water (BPW), according to the protocol by Houben (2005). This dilution was mixed using a Stomacher (Colworth Stomacher 400, Stomacher Lab System Model 400 bags, Seward, London, UK) for 120 s. Decimal dilutions were made to create countable dilutions. Specific department protocols were based on ISO methods and the textbook *Microbiology of Foodstuffs* by Dijk and Grootenhuis (editors, 2003) for further bacterial isolation and confirmation. Since monocultures were used for the inoculations, less selective media could be used to provide better yields and more accurate cell counts. The selected ISO methods and media used for plating and enrichment are given in Table 2.
The first step in the analysis of *C. perfringens* spores was the heat treatment of the tubes containing the first dilution (in BPW) to eliminate vegetative cells. A thermostatically controlled water bath was used at 75±0.1 °C for exactly 15 min

after the temperature in a control tube reached 75 °C. According to Houben (2005) the Iron Sulphite Agar (ISA) was a suitable medium for *Clostridium* spores in casings and therefore incorporated into our protocols.

Statistical analysis

According to Lenovich (1987), the graphic illustration of microbial survival can be obtained by plotting the logarithm of survivors against time. Since these plots are generally linear, survival curves can be described by linear correlations. The slopes of the survival curves were calculated using regression-analysis. The absolute values of these slopes were defined as death rates (day^{-1}). The statistical analyses were carried out using SPSS Version 12.0.1 software (SPSS, IL, USA).

Results and discussion

The brine concentrations and dry salt samples were measured repeatedly for their water activity, giving a_w-levels of 0.90 a_w, 0.87 a_w, 0.85 a_w, 0.83 a_w and 0.75 a_w respectively. The survival characteristics of the non-spore forming bacteria at these a_w-levels are presented in Table 3.

Bacterial species	a_w-levels				
	0.90	**0.87**	**0.85**	**0.83**	**0.75**
E. coli	0.11[a]	0.25	0.41	0.26	1.50
E. coli O157:H7	0.10	0.15	0.16	0.38	0.41
S. typhimurium	0.17	0.16	0.34	0.32	0.34
L. monocytogenes	0.20	0.10	0.10	0.10	0.26
S. aureus	0.11	0.11	0.11	0.11	0.11

a) Death rate (log_{10} cfu day^{-1}),

Table 3: reduced survival of bacterial species in natural casings at different a_w-levels

Overall it can be stated that Gram-positive bacteria are less sensitive to lowered a_w than Gram-negative bacteria. This is consistent with the fact that Gram-positive bacteria are better equipped to cope with osmotic stress than Gram-negatives (Mellefont et al., 2003). Gram-positive bacteria have constitutive transport systems for the uptake of compatible solutes, whereas Gram-negatives need to implement transport systems (Mellefont et al., 2003). Until this implementation has taken place, accumulation of potassium and its counterion glutamate is used to maintain the turgor (Mellefont et al., 2003; Sleator et al., 2003). The accumulation results in high intracellular levels of potassium glutamate, possibly impairing enzyme function. Furthermore, Gram-negative bacteria display a relatively low turgor, giving a more severe plasmolysis during osmotic stress (Gutierrez et al., 1995). This impairment of enzymes together with the more severe plasmolysis gives an explanation for the higher death rates observed for Gram-negative bacteria at lower a_w.

As illustrated in the introduction, there are very few studies done on the survival of pathogens in natural casings preserved in salt. Only the study by Gabis and Silliker (1974) on the survival of *Salmonella spp*. showed a decrease of approximately one log-cycle in three days in saturated brine (0.75 a_w). A death rate of 0.33 day^{-1} can be calculated, which corresponds to our findings. In recent years, the survival of *E. coli O157:H7* in many other foodstuffs has been the subject of several studies. Rocelle et al. (1996) showed a decrease of

approximately 0.14 $\log_{10}$ cfu g^{-1} day^{-1} at 20 °C and 0.90 a_w in salami and Park and Beuchat (2000) showed a decrease of approximately 4.40 $\log_{10}$ cfu g^{-1} $week^{-1}$ in starch. These values are higher than the death rates found in this study which could be explained by the relatively low inoculation levels used for *E. coli O157:H7* and the differences in composition of the foods used in the different models (Park and Beuchat, 2000).

A study on the survival of *L. monocytogenes*, (Nolan et al., 1992) recorded a reduction of five log-cycles in seven days at 0.91 a_w in TSB-YE and added sodium chloride. This is a markedly higher death rate than was observed in our study, but the data from the Nolan study were obtained at a_w levels in the bacterial growth range whereas lower a_w ranges as reported by Uzelac and Stille (1977), will increase survival. These findings correspond to our findings where *L. monocytogenes* survived for up to 30 days at an a_w-level of 0.85.

Death rates of *S. aureus* were constant for all investigated a_w-levels. *S. aureus* is known to be the most halotolerant non-halophilic eubacterium, and can grow at a_w-values as low as 0.86 (Gutierrez et al., 1995; Abee and Wouters, 1999). This is caused by the highly effective transport systems of *S. aureus* for compatible solutes. Nevertheless, a severe decrease in viable cells was observed over the 30-day period. The reason for this subsequent cell-death could be found in a shortage of compatible solutes or substrate for compatible solutes, since *S. aureus* needs exogenous supply of these substances (Gutierrez et al., 1995). Shortage of energy can be another factor leading to death of the bacteria, since many mechanisms required to maintain turgor are energy-dependent (Verheul et al., 1997; Abee and Wouters, 1999).

No clear reduction in the survival of *C. perfringens* in the relation to any a_w level was observed. After inoculation the vast majority of cells died, probably due to the presence of oxygen, since *C. perfringens* is an obligatory anaerobe and very sensitive to the presence of oxygen (Trinh et al., 2000). This left only a few cells to sporulate, which were re-grown using the enrichment procedures as described in Table 2. Due to the fact that *C. perfringens* is a natural inhabitant of the intestinal tract of many animals (Brynestad 2002), the *Clostridium* spores were not only found during the casing processing (Ockerman and Hansen, 2000), but their presence was also confirmed in salted natural casings, sometimes at high concentrations (Houben, 2005). The recurring re-growth of bacterial spores as found in our study (results not shown) clearly demonstrates the resistance to low a_w-levels of *Clostridium* spores.

In this study, cultures were diluted in TSB, prior to insertion of the inoculum into the casing samples. Since one of the ingredients of TSB is soy-peptone, a known substrate for several compatible solutes (Sleator et al., 2003), this may have had a protective effect on the bacteria. The presence of soy-peptone may have therefore artificially increased the survivability of the bacteria.

The temperature at which the casings were stored (20±1.5 °C) was within the range that provides the highest tolerance against osmotic stress (Mellefont et al., 2003). The measured pH was close to neutral during the storage of the casings and no detrimental influence was to be expected. The results found in this study may therefore represent the maximal survival of specific pathogens in salt-preserved casings. With the exception for *L. monocytogenes*, all non-spore forming bacteria could not be positively identified after the mandatory 30-day preservation period for casings at a water activity level of 0.85 or lower (Figure 1).

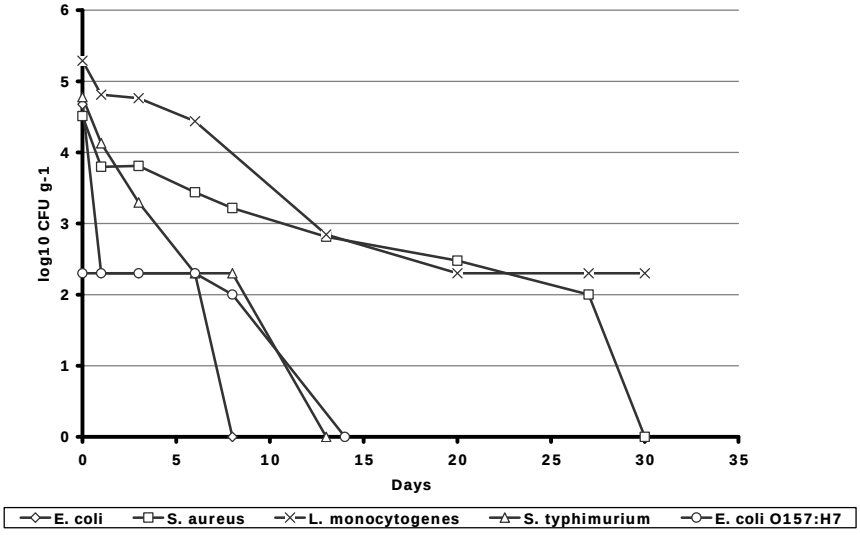

Figure 1: Bacterial reduction over time at a water activity level of 0.85

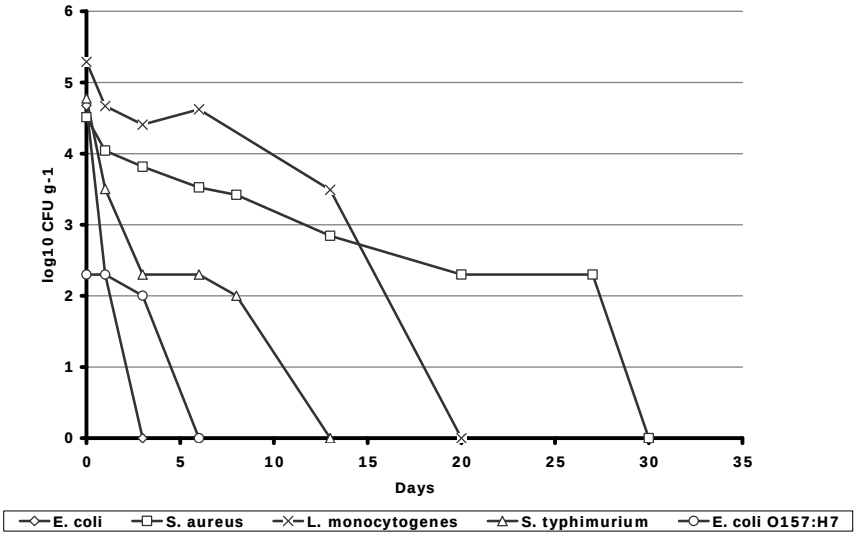

Figure 2: Bacterial reduction over time at a water activity level of 0.75

However, at a water activity level of 0.75, *L. monocytogenes* could not be positively identified after 20 days. Similar to the findings of Gabis and Silliker (1974), *S. typhimurium* was no longer present in dry-salted casings after 13 days. In general the casing industry uses either dry salt (a_w-level 0.75) or saturated brine (a_w-level between 0.75 and 0.80) for preservation and the storage period will exceed the minimum required 30-day period. This way a clear safety margin exists for all non-spore forming bacteria to be well below any microbiological critical limit currently in use for preserved natural casings (Table 1).

Conclusions

The results found in this study can be directly applicable to the casing industry and may support the known antimicrobial properties of salt used in the traditional methods for the preservation of natural sausage casings. The inoculation levels used reflect the actual bacterial counts during the cleaning process of unsalted casings and the casing model used is an acceptable representative for the preservation of casings. By measuring the water activity level at which no bacteria could be found or where bacteria were below any critical limit, a novel parameter for salt-preserved natural casings becomes available during routine Quality Control Inspection and monitoring of the preservation process.
All findings from this study comply with the hurdle concept described in detail by Leistner and colleagues (Leistner, 2000, review). Specific findings, for instance the survival of Clostridium spores, show that the hurdles established during the processing of casings and subsequent curing and storage in dry salt or brine, are not completely effective to eliminate all remaining contamination. This situation will require continuous attention and will lead to further research on the optimal preservation of natural sausage casings.

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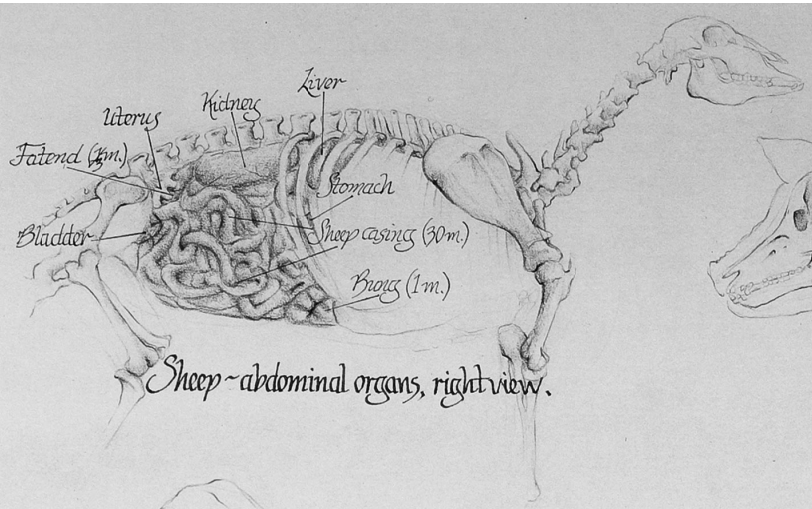
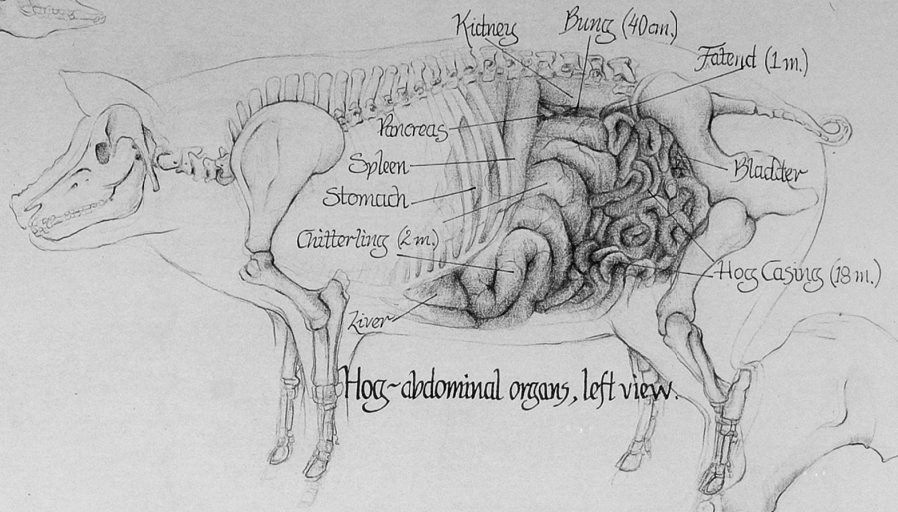
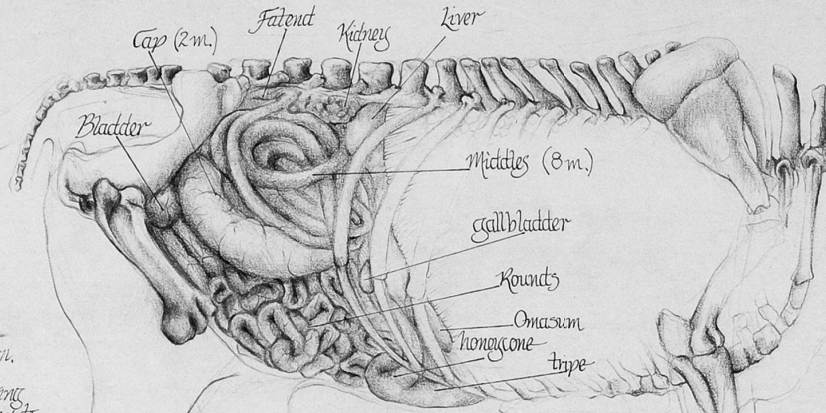


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 3

Reduction of *Clostridium sporogenes* spore outgrowth in natural sausage casings using nisin

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Abstract

Preservation of natural sausage casings using dry salt or saturated brine is regarded as sufficient to inactivate vegetative pathogenic non-sporeforming bacteria present on the casings. This salt or saturated brine preservation does not prevent outgrowth of bacterial spores. To prevent this outgrowth additional preservation measures should be implemented. In the experiments described the use of nisin was evaluated to reduce outgrowth of spores in desalinated casings. The bacteriocin nisin was chosen because of its known efficacy against spore-forming bacteria and their spores in various food stuffs. *Clostridium* spore suspensions (*C. sporogenes*, ATCC 3584) were used in 2 concentrations to inoculate 3 nisin concentrations (10, 50, 100 µg / ml) in water containing gamma-irradiated casings. Additionally, the binding of nisin to casings, using ^{14}C-labeled nisin Z and subsequent bioavailability of nisin were evaluated. Results demonstrate that nisin is bound to casings but if sufficient nisin was present the outgrowth of *Clostridium* spores in this model was reduced. However, the biological relevance of these results needs to be determined further by conducting industrial trials before any recommendation can be made on the practical implementation of nisin in the preservation of casings.

Introduction

Preservation of animal intestines as edible casings of sausages has been practiced for centuries with clear antimicrobial effects and its application described throughout history (Savic and Savic, 2002). Although the processing of intestines into natural sausage casings has been modernised over time (Ockerman and Hansen, 2000), the preservation still relies on salt to allow prolonged storage without refrigeration (Fischer and Schweflinghaus, 1988a, 1998b). A study by Gabis and Silliker (1974) showed the efficacy of salt against *Salmonella* in casings, whereas a study by Wijnker et al. (2006) indicated that all pathogenic non-sporeforming vegetative bacteria tested were no longer present after a 30 days storage period at ambient temperatures, using either dry salt or brine (water activity level < 0.83). However, *Clostridium perfringens* spores and other sulphite reducing anaerobic spores remained viable after prolonged storage in salt (Wirth 1990, 1994; Houben 2005; Wijnker et al., 2006). Subsequent processing of sausages, such as pasteurisation (Levison and Feeherry, 1978) or an increase in water activity (Zaika, 2003) can trigger bacterial spores to return to their vegetative state causing major health risks for the final consumer (Shandera et al., 1983; Craven and Blankenship, 1985; McClane, 1992; Mead et al., 1999).

Clostridia are ubiquitously present in the environment and in the intestinal flora of both man and animals (International Commission on Microbiological Specifications for Foods, 1996). High spore counts (> 1000 cfu / g casing) have been found both in freshly produced unsalted sheep casings (Koolmees and Houben, 1997) and dry salted hog and sheep casings (Houben, 2005). The goal of this study was to determine if the preservation of casings can be augmented to reduce the presence and subsequent outgrowth of sulphite-reducing *Clostridia* spores.

A likely substance to achieve this effect is nisin, a bacteriocin produced by certain strains of *Lactococcus lactis subsp. lactis* (Mattick and Hirsh, 1947; Schleifer et al., 1985), with a known broad-spectrum inhibitory effect on gram-positive bacteria, including spore-forming bacteria and their spores (Hurst, 1981; Davies et al., 1999).

Nisin is able to kill bacteria via targeted pore formation after developing a stable complex with Lipid II, an essential intermediate in the bacterial cell-wall synthesis, causing leakage of potassium ions and intracellular metabolites such as ATP (Breukink et al., 1999; Hsu et al., 2004)

The affected bacteria die as a result of energy depletion and stagnation of intracellular biosynthetic processes (Ruhr and Sahl, 1985; Sahl and Bierbaum, 1998).

The effect of nisin against spores is more likely to be sporostatic than sporocidal (Delves-Broughton et al., 1996), and has been suggested to be the result of binding to sulfhydryl groups on protein residues (Ando, 1979, Morris et al., 1984). Meghrous et al. (1999) recorded a 10-day delay in *Clostridium* spore outgrowth after exposure to nisin, whereas sporocidal effects of nisin have been observed by De Vuyst and Vandamme (1994) after spores were damaged by heat treatment (3 minutes at 121 °C).

Nisin is most soluble in acidic substrates and becomes progressively less soluble at neutral or alkaline pH (Liu and Hansen, 1990). Nisin can also absorb to proteins in the food matrix by ionic or hydrophobic bonds (Coventry et al., 1995; Aasen et al., 2003) reducing its bio-availability for bacteria and spores.

In this study, the use of nisin was evaluated to reduce outgrowth of spores by developing a suitable model using casings. To this purpose casings were exposed to gamma radiation at 10 kGy as studies by Trigo and Fraqueza (1998) and Chawla et al. (2006) had already identified this radiation dose to be sufficient for the destruction of all *Clostridium* spores and vegetative bacteria in casings. Although it is an effective method, it is not allowed for casings in Europe (European Parliament and Council, 1999).
Taking into account the biochemical properties of nisin, the following questions were to be answered: 1) can nisin reduce the outgrowth of *Clostridia* spores present in casings; 2) does nisin bind to casings or to spores; 3) if nisin is bound to casings can it remain biologically available for antimicrobial action.
The outcome of this study may lead to better ways to treat sausage casings for the reduction of *Clostridium* spore outgrowth.

Materials and methods

Clostridium strain

For safety reasons, *C. sporogenes* was chosen as model organism in these studies as it is regarded as a safe and suitable substitute for *C. botulinum* (Montville et al., 1985). *C. sporogenes* (ATCC 3584) spore suspensions were obtained in triplicate from TNO Quality of Life (Zeist, the Netherlands). Of each suspension the final concentration of spores was counted allowing inoculations of approximately 5000 and 50.000 spores per gram casing.

Casings

Dry salted Australian sheep casings (AA 18-20), were desalinated, checked for holes, divided into 1 meter strands (weight approximately 7 g / m casing), bundled and stored in saturated brine (water activity level < 0.79). These bundles were subsequently exposed to gamma radiation (Cobalt-60, 10 kGy, Isotron Nederland BV, Etten-Leur, The Netherlands) while in a closed container.
To one irradiated casing strand, 100 ml of buffered peptone water (PBM) was added in a stomacher bag and mixed for 120 seconds to dislodge any bacteria or spores from the casing wall and create a homogenous suspension (Colworth Stomacher 400, Stomacher Lab System Model 400 bags, Seward, London, UK). Two decimal dilutions of the suspension were made and all were used according to a five-tube Most Probable Number (MPN) table to determine any residual bacterial presence (Halvorson and Ziegler, 1933). From the original suspension and two dilutions 0.1 ml of fluid was added to five 10 ml Tryptone Soy Broth tubes (Ref. no. CM 0129, Oxoid Ltd, Basingstoke, UK) each and incubated for 72 hours at 30 °C under aerobic conditions. In addition, five Tryptone Soy Agar plates (Ref. no. CM 0131B, Oxoid Ltd, Basingstoke, UK) and five Schaedler agar plates (Ref. no. 43401, bioMérieux, Marcy l'Etoile, France) were inoculated with either suspension or dilutions before and after heating in a water bath (10 min, 80 °C). All plates were incubated for 72 hours at 30 °C, while the Schaedler agar plates were stored under anaerobic conditions using a commercial gas pack (GasPak™ EZ Anaerobic Container System, Cat. No. 260678, Becton Dickinson).

Inoculation and microbial analysis

Three preservation solutions were made using sterile demineralised water and nisin (Nisaplin, containing 2.5% nisin A, Danisco, Copenhagen, Denmark) at a concentration of 10, 50 and 100 μg / ml and the pH was set to 5.7, 5.1 and 4.8

respectively (Mettler-Toledo SevenEasy digital pH meter, Mettler-Toledo B.V., Tiel, the Netherlands). Centrifuge tubes (50 ml, Corning, NY, USA, item no. 430829) were filled with 20 ml preservation solution (30 tubes per solution) or with 20 ml sterile demineralised water for the negative and positive controls. Each 1 m strand of casing was desalinated in sterile demineralised water, cut open lengthwise under sterile conditions and 20 cm sections were added to each of the prepared sample tubes. Final inoculation with the *C. sporogenes* spore suspension was done according to the matrix in table 1.

Series	Inoculation cfu[a] / g casing	Nisin conc. mg / l	No. casing samples per series	No. tubes
Neg. Ctrl.	0	0	3	15
Pos. Ctrl.	50.000	0	3	15
1	5000	10	3	15
2	5000	50	3	15
3	5000	100	3	15
4	50.000	10	3	15
5	50.000	50	3	15
6	50.000	100	3	15

a) colony forming unit

Table 1: inoculation matrix *C. sporogenes* spores and nisin concentration

All samples were gently shaken and stored in the dark at ambient temperatures.

Microbial analysis

On day 1, 8, 15, 22 and 29 after inoculation, three sample tubes of each series were taken for microbial analysis. The content of each tube was transferred to a stomacher bag and mixed for 120 seconds to dislodge any spores from the casing wall and create a homogenous solution (Colworth Stomacher 400, Stomacher Lab System Model 400 bags, Seward, London, UK). Fluid samples from each bag were taken in duplicate (0.1 ml) before and after heating in a water bath (80 °C for 10 minutes) to inoculate Schaedler agar plates for bacterial counts. Agar plates were subsequently covered with 10 ml Schaedler agar (Ref. no. CM0437B, Oxoid Ltd, Basingstoke, UK) and incubated under anaerobic conditions for 48 hours at 30 ºC. The specific colony form of *C. sporogenes* was confirmed and final plate counts were made. The number of colony forming units (cfu) per gram casing was calculated by multiplying the cfu counted with a factor of 200.

Additional analyses

The specific binding of nisin to casings or *C. sporogenes* spores was studied qualitatively by using a mixture of nisin A and ^{14}C-labeled nisin Z (Breukink et al., 1997), which is a natural variant of nisin A that only differs in one single residue and possesses almost identical antimicrobial properties (De Vos et al., 1993).

Four centrifuge tubes (15 ml, Corning, NY, USA, item no. 430791) were filled with 1 ml 10 μg / ml nisin solution (8 μg nisin A, 2 μg, ^{14}C-labeled nisin Z at 28.000 dpm[1] / nmol). To each tube a section of 4 cm^2 of desalinated casing and 1.10^5 cfu *C. sporogenes* spores were added. Tubes were vortexed and left to

[1] dpm = disintegrations per minute

incubate at room temperature for 4 (group 1) or 16 hours (group 2). Each tube was centrifuged for 10 minutes at 4000g to spin down a spore- pellet, allowing the contents of the tube to be split into three fractions: casing, fluid and pellet. Each fraction was subsequently analysed using a liquid scintillation counter (Tri-carb 1500, Packard Instrument Company, Downers Grove IL, USA) to determine the presence of ^{14}C-labeled nisin Z.
The bioavailability of nisin bound to casings was determined by comparing bacterial growth inhibitory zones surrounding nisin impregnated casing and paper disks (No. 2668, diameter 12.7 mm, Schleicher & Schull**,** Dassel, Germany) placed on poured Schaedler agar plates, inoculated with sufficient *C. sporogenes* spores or vegetative cells to produce confluent growth.
Uniform casing disks (diameter 12.7 mm) were prepared from desalinated casings using a handmade stainless steel punch in an industrial bench press.
The existing stock of *C. sporogenes* spore suspension (ATCC 3584) was checked for viability, plated on Schaedler agar plates and counted. Tubes filled with liquid Schaedler agar were inoculated with 1 ml spore suspension or vegetative cell culture (final volume 9 ml), poured into plates and left to solidify.
Three casing or paper disks were placed per plate on the inoculated agar and 20 μl of a 1, 5, 10, 50 or 100 μg / ml nisin solution in sterile demineralised water was added onto the disks. All nisin concentrations were prepared in triplicate and negative controls added for each concentration. Plates were incubated under anaerobic conditions for up to 72 hours at 30 ºC and checked for the development of inhibitory zones at 24 hour intervals.

Statistical analyses

The *C. sporogenes* cfu counts were likely to follow a Poisson distribution but due to overdispersion in the positive control group and the first time point (day 1), a negative binomial distribution was taken with a scale parameter of 0.01. A generalized linear mixed effect model was performed (Bates, 2007) in R version 2.7.0 (R Development Core Team, 2008) with the actual counted cfu (not taking into account the dilution factor) as the outcome. The variable “sample” was taken as the random effect because from each sample 2 cfu were counted. The explanatory categorical variables are the nisin concentration used (10, 50 and 100 μg / ml), whether the samples were heated in a water bath (yes / no), time point for analysis (Day 1, 8, 15, 22 or 29) and amount of *C. sporogenes* spores used for inoculation (5000 or 50.000). The positive control group with a nisin concentration of 0 μg / ml was only combined with spores equal to 50.000. Therefore the difference of the nisin concentration 0 with dose 10, 50 and 100 was analyzed using only the observations with spores equal to 50.000 with nisin, bath and time as explanatory variables. In both models 2-way interactions between the explanatory variables were also tested by the likelihood ratio test with 0.05 as the significance level.

Results

Irradiated casings

The results from the MPN analysis on the irradiated casings indicated that the residual (spore) contamination of the casings, if any, was very low (MPN value < 0.01) and all negative control samples (no spores inoculated, no nisin added) included in the *C. sporogenes* spore inactivation experiment, confirmed this outcome.

Microbial analysis

In order to confirm reproducibility of the model the experiment was started in three consecutive weeks following the same protocol. Similar patterns in the results were identified allowing all corresponding groups to be combined for further analysis.

The descriptive results (plate counts X dilution factor 200) presented in figures 1 and 2 show the relation between the numbers of *C. sporogenes* cfu found and the respective time points. The median cfu values found on each time point are represented by the horizontal bar in each box plot, while individual values over 1.5 times the interquartile range (IQR) or 3 IQR's are represented as outliers (^{o}) or extremes (*) above the whiskers. Each box plot represents all measurements taken for the 10, 50 and 100 µg / ml nisin concentrations. Box plots from each nisin concentration are combined as all show a similar pattern. These results already indicate that a significant difference in cfu can be found between day 1 and day 8, while cfu found on day 15, 22 and 29 may not differ from day 8.

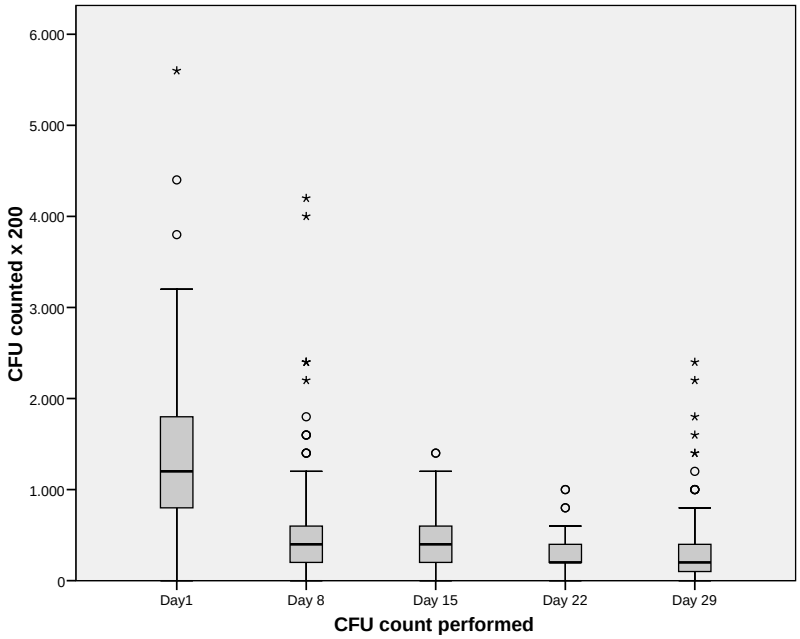


Figure 1: Relation between cfu counts from all treatment groups and their respective time point (5.10^3 spores inoculated)

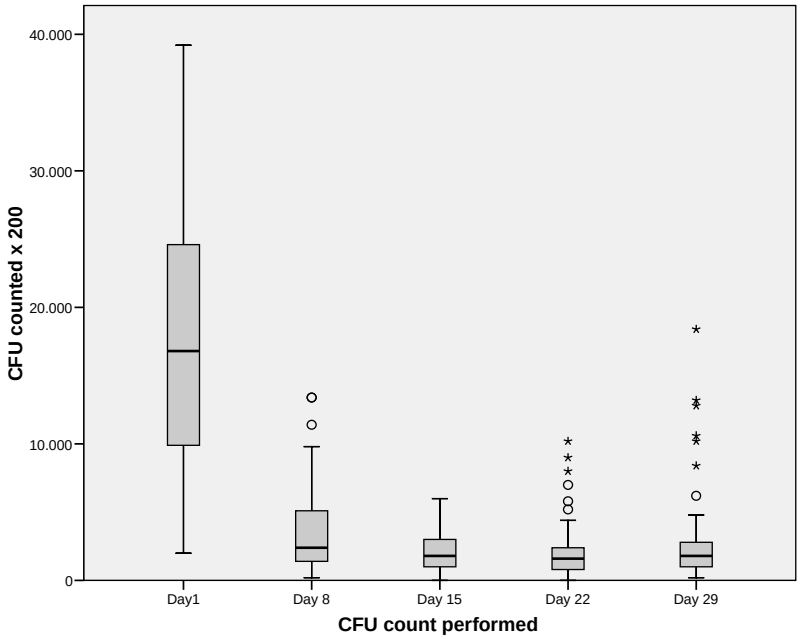


Figure 2: Relation between cfu counts from all treatment groups and their respective time point (5.10^4 spores inoculated, excluding pos. ctrl.)

Table 2 shows the number of *C. sporogenes* cfu (x 200 as dilution factor) found in the respective treatment groups at day 1 and day 8 after inoculation.

Nisin (μg / ml)	Before / after water bath	Time point	No. spores inoculated	cfu x 200 (mean)	Standard Deviation
0 (pos. ctrl.)	Before	Day 1	5.10^4	34.333	7124
		Day 8	5.10^4	28700	6989
	After	Day 1	5.10^4	22400	6700
		Day 8	5.10^4	17122	7432
10	Before	Day 1	5.10^3	2511	855
		Day 1	5.10^4	28533	7765
		Day 8	5.10^3	967	818
		Day 8	5.10^4	5633	2017
	After	Day 1	5.10^3	2050	874
		Day 1	5.10^4	26756	8086
		Day 8	5.10^3	789	728
		Day 8	5.10^4	4150	2292
50	Before	Day 1	5.10^3	1389	663
		Day 1	5.10^4	14711	6321
		Day 8	5.10^3	922	1245
		Day 8	5.10^4	4211	3307
	After	Day 1	5.10^3	978	531
		Day 1	5.10^4	12300	7580
		Day 8	5.10^3	311	230
		Day 8	5.10^4	1478	1043
100	Before	Day 1	5.10^3	1533	1168
		Day 1	5.10^4	14611	6531
		Day 8	5.10^3	267	247
		Day 8	5.10^4	5100	4163
	After	Day 1	5.10^3	722	344
		Day 1	5.10^4	10456	5268
		Day 8	5.10^3	289	268
		Day 8	5.10^4	1922	1348

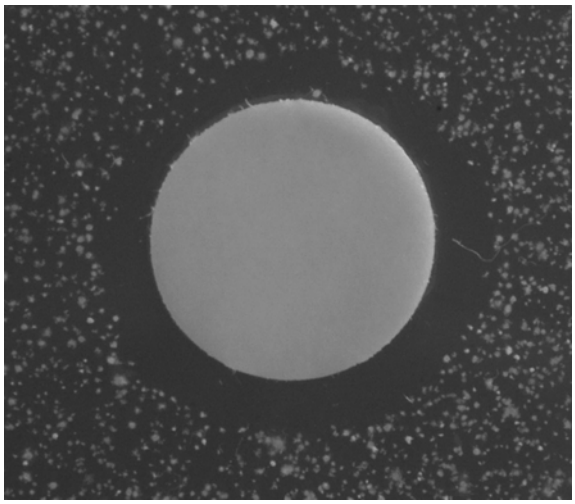
Table 2: Number of *C. sporogenes* cfu in various treatment groups at day 1 and day 8 after inoculation.

Based on the generalized linear mixed effect model (actual counted cfu, not taking into account the dilution factor) the following results were found: There were no interaction effects between the four explanatory categorical variables present in the model ($1 > P > 0.92$); The average cfu found on Day 8 were 0.26 x the average cfu found on Day 1 (significant decrease, $P < 0.001$); The average cfu found after treatment with 50 μg / ml were 0.62 x the average cfu found after treatment with 10 μg / ml (significant decrease, $P < 0.001$); The average cfu found after the water bath heat treatment were 0.66 x the average cfu found before the water bath heat treatment (significant decrease, $P < 0.001$); The average cfu found after inoculation with 5.10^4 spores were 7.11 x the average cfu found after inoculation with 5.10^3 spores (significant higher level, $P < 0.001$); There were no significant differences between cfu found at Day 8 and Day 15, Day 22 and Day 29; There was no significant difference found between treatment with 50 μg / ml and 100 μg / ml.

Binding and bioavailability of nisin

Analyses of the three fractions -casing-, -fluid- and -pellet- indicated that ^{14}C-labeled nisin Z was predominantly present in the casing fraction (results not shown), suggesting a firm binding between nisin and the casing wall.

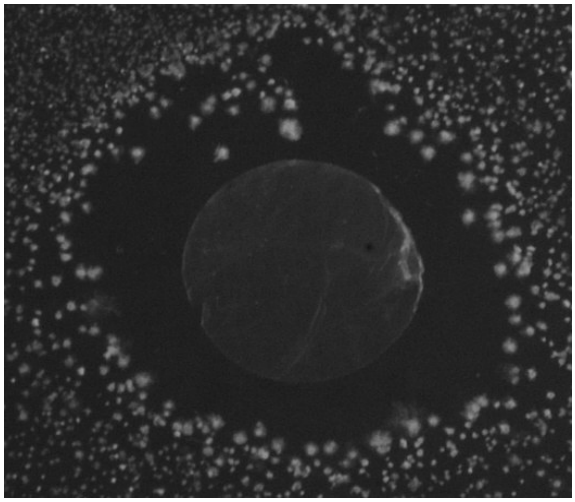
In the bioavailability assay, clear circular inhibitory zones surrounding all paper disks (diameter 2-3 mm, *n* = 18 per conc.) were found after 48 hours of incubation when a nisin concentration of 50 and 100 μg / ml was used (Picture 1).



Picture 1: Inhibitory zone paper disk

Inhibitory zones were also found surrounding all casing disks (*n* = 18 per conc.) at a nisin concentration of 100 μg / ml. As with the paper disks, these zones were found after 48 hours of incubation (Picture 2), but were irregularly shaped (diameter 1-5 mm).

No differences were found between inhibitory zones for plates inoculated with spores or vegetative bacteria and the size of the zones did not increase further after 48 hours of incubation.



Picture 2: Inhibitory zone casing disk

Discussion

In this experiment gamma irradiation is used to destroy all bacteria and spores present on the casings used. The usability of gamma irradiation at 10 kGy depended not only on the efficacy to destroy microbial contamination to create a “clean” model but also whether this treatment would damage the casing beyond practical use. Different casing samples taken after gamma irradiation were tested on various quality parameters, which revealed no detrimental effect of the treatment. As a result it can be concluded that gamma irradiation at 10 kGy can effectively inactivate any bacteria or spores present on the casings without quality loss.

Absence of any significant differences between cfu found on day 8, 15, 22 and 29 suggest that a maximum sporostatic activity was reached between day 1 and day 8 using a nisin concentration of 50 µg / ml, as similar results were found for the 100 µg / ml solution. This 30 day delay in spore outgrowth was substantially longer than the 10 day delay previously recorded by Meghrous et al. (1999) for several *Clostridia* strains including *C. sporogenes*, but it remains to be determined what the full inhibitory period will be.

Bacterial spores are very resistant to heat and survive many cooking and processing treatments. Transformation of these spores to actively growing

vegetative cells is a three stage process: activation, germination, and outgrowth. Nisin will not affect germination but prevents post-germination swelling and subsequent spore outgrowth (Thomas et al., 2000). Heat often serves to activate the spores and they will subsequently germinate and grow if conditions are favourable. In this respect, the differences between results before and after the water bath treatment (80 °C, 10 min.) may appear illogical, as lower counts were found after the heat treatment. A possible explanation is that some spores had already germinated prior to the heat treatment independent of the presence or absence of nisin and were already in a vegetative state. The water bath heat treatment would have inactivated these bacteria and subsequently reducing the number of cfu in the final count.

The results from the binding and bioavailability study using the ^{14}C-labeled nisin Z and nisin impregnated disks indicate that nisin is bound to the casing wall, whilst exhibiting bactericidal activity away from the casing wall. Previous studies have clarified that nisin binds to glutathione (GSH) in a non-enzymatic reaction (Stergiou et al., 2006), with the free sulfhydryl group of GSH as the proposed binding site for nisin (Rose et al., 1999). In addition it has been shown that the inhibiting mechanism for spore outgrowth is also due to nisin binding to sulfhydryl groups in the envelopes of germinating spores (Morris et al., 1984; Liu and Hansen, 1990). It is therefore quite likely that the sulfhydryl groups in the casing wall and on the spore surface are in competition to bind the available nisin.

As said, the results from the bioavailability study show bactericidal activity away from the casing wall, by producing inhibitory zones around the casing disks. However, it is unclear whether this was achieved by nisin that was bound reversibly to the casing wall and subsequently released or by unbound nisin that diffused unhampered away from the casing disk as all sulfhydryl groups were already occupied. Assuming the latter, a nisin concentration is required above the Minimal Inhibitory Concentration for *C. sporogenes* (38.4 µg / ml, Meghrous et al., 1999), combined with the amount needed to saturate the sulfhydryl groups in the casing disk. Absence of any inhibitory zones surrounding the paper disks at nisin concentrations lower than 50 µg / ml and only an inhibitory zone surrounding the casing disks at the highest nisin concentration (100 µg / ml) are in line with this assumption.

Based on the different parameters included in this study (nisin concentration, water bath, time point, amount of spores used) an optimum effect can be determined. This effect is defined by the spore count x available nisin x exposure time. The additional handling of the samples by placing them in a hot water bath can not be taken into account as it will ruin the usability of the casing. As there were no interactions between the explanatory variables, the optimum effect is independent of the amount of spores used for the inoculation. An optimum effect is achieved when the casings have been exposed to a 50 µg / ml nisin solution for a period of 8 days. If the original inoculation level is used and compared to the related cfu counts, a spore reduction of approximately 1 $\log_{10}$ is achieved.

In this experiment it was demonstrated that nisin can augment the preservation of casings to reduce the outgrowth of spores present on casings. Empirical data supports these findings which may warrant additional industrial trials to determine whether a practical application of nisin in the preservation of casings can be recommended.

Acknowledgments

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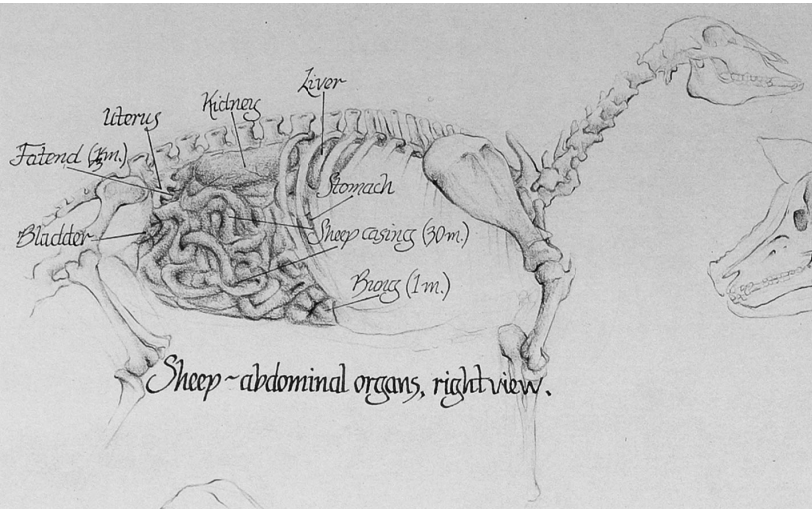
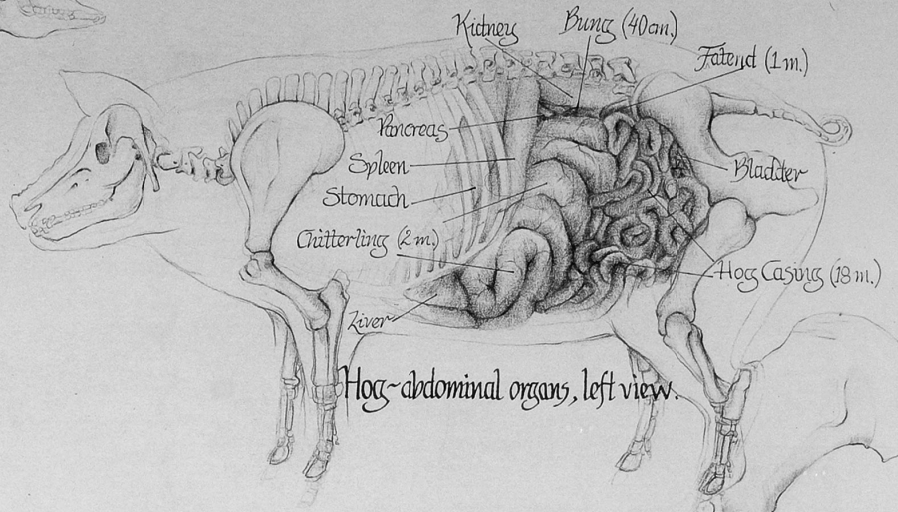
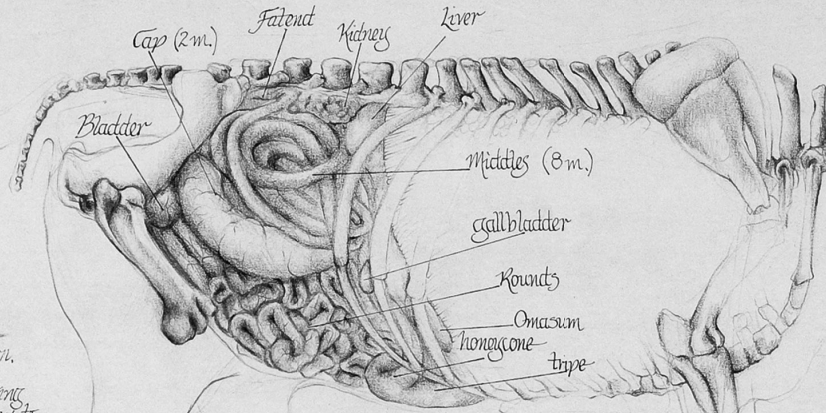


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER IV

Quantitative histological analysis of bovine small intestines before and after processing into natural sausage casings

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Abstract

A histological study was undertaken to determine the efficacy in the removal of the mucosa and Peyer's patches by standard processing of bovine intestines into natural sausage casings. The second objective was to calculate the quantity of lymphoid and nervous tissue per consumable sausage. For the histological analysis, intestinal samples were collected from 80 beef cattle during the slaughter process. Fresh and cleaned intestines were compared in analyzing the thickness of the intestinal wall, weight reduction during cleaning, removal of the mucosal layer, and the presence of lymphoid and neural tissue after cleaning. The obtained data indicate a weight reduction of about 50% during standard cleaning procedures, as 90% of the mucosa and 48% of the lymphoid tissue are removed. Based on the quantitative histological image analysis, it was calculated that 1 m of cleaned casings, weighing on average 64 g, contains about 2.8 g of mucosa, 0.3 g of lymphoid tissue, and 0.1 g of neural tissue. Assuming, in a worst-case scenario, that the sausage casing is ingested when consuming 200 g of sausage at one meal, this consumption includes 0.09 g of lymphoid tissue and 0.02 g of neural tissue as part of the sausage casing. These data can be included in a risk assessment on the potential exposure of consumers to bovine spongiform encephalopathy infectivity after eating sausages in beef casings.

Introduction

Processed intestines from pigs, sheep, and cattle are used as edible natural casings for the production of sausages. Given the large variety in sausages, specific qualities and calibres of casings are required from all species mentioned (Table 1). Unlike sheep and hog casings, beef casings are normally removed prior to consumption of the sausages (Savic and Savic, 2002), as they are thicker, tougher (similar to tendon), and therefore practically unpalatable. However, as consumption cannot be excluded entirely, an estimate on the presence of tissues related to the bovine spongiform encephalopathy (BSE) exposure risk in beef casings is warranted. Based on available import data for 2005 (Eurostat, 2007), it can be estimated that the beef casings used in Europe for the production of sausages amount to approximately €87 million, with a volume of 68,000 metric tons.

Table 1: Examples of sausages and their natural casings

Type of casing used	Sausages	
Beef casing	Blood sausage	Polish sausage
	Liver sausage	Ring Bologna
	Chorizo	Holsteiner
	Mettwurst	Kishka
Hog casing	Pepperoni	Polish sausage
	Bratwurst	Italian sausage
	Chorizo	Pork sausage
	Kielbasa	Linguisa
	Smoked sausage	Frankfurters
	Dinner Franks	Knockwurst
Sheep casing	Wieners	Hot dogs
	Franks	Pork sausage
	Cocktail wieners	Mortadella

Source: www.casings.com

Since BSE was first officially diagnosed in the United Kingdom in November 1986, drastic measures have been taken by the European Union authorities to ensure consumers' safety. Among others, beef casings produced from European bovine intestines were designated specified risk material, and were banned from human consumption completely in 1997 (European Commission Scientific Steering Committee, 1997, 2000). However, BSE infectivity has so far only been confirmed in the distal ileum and not in any part of the intestinal tract used for the production of beef casings (Wells et al., 1994; Terry et al., 2003; Buschmann and Groschup, 2005; Hoffman et al., 2007). This precautionary ban terminated the production of beef casings in Europe and allowed only imports into the European Union from countries with a negligible BSE risk (European Commission, 2001).

In order to provide data for an objective risk assessment on the potential BSE infectivity of beef casings, this study aimed to analyze by means of quantitative histological methods, the cleaning steps generally used in the processing of bovine intestines into natural casings. Together with available data on tissue distribution of BSE infectivity, the steady decline of BSE notifications in Europe and implemented feed ban (since 2001), these data will provide for some of the rationales for a quantitative risk estimate.

Materials and methods

Processing and sampling

Intestinal samples were obtained during normal slaughtering processes from four South American slaughterhouses (two each in Brazil and Uruguay) approved for export to the European Union. Intestines were subjected to veterinary inspection and were regarded suitable for human consumption. All slaughterhouses have on-site facilities for the processing of bovine intestines, which is done according to standardized operating procedures, which resemble closely the cleaning procedures used in Europe.

In Brazil, Nelore cattle were used (all male, average body weight and age: 360 kg and 3.5 years, respectively), and in Uruguay, Hereford cattle were used (all male, average body weight and age: 500 kg and 2.8 years, respectively). All cattle (n = 80) were randomly selected at the slaughter line and corresponding gastrointestinal tracts labelled for subsequent sample identification. Samples were taken from the intestines at two fixed moments during the processing, representing fresh intestines and cleaned casings. An identical sampling protocol was used at each facility to ensure uniformity.

When post-mortem inspection was completed, the intestines were separated from the stomachs and transferred to the gut room for immediate processing according to standardized operating procedures. The intestines were hand pulled from the mesentery and cut off with a knife approximately 2 m proximal to the ileocecal junction, forming 80 sets of intestines comprising the duodenum and most of the jejunum. The remainder of the small intestines, comprising the entire ileum and a small part of the jejunum, were removed and incinerated.

After hand pulling, the outside of each set was scraped manually to remove any remaining mesentery, adipose, or loose tissue. Each set was tagged at the stomach end for identification, and manure was stripped mechanically from the lumen. From the original 80 sets, half were used after this processing step for sampling and denoted as fresh intestines.

To obtain samples representing cleaned casings, the remaining 40 sets were turned inside out (standard operating procedure) and transferred to a Stridhs-type cleaning machine (Mecanica Primitiva, Ltda, Salto Grande, São Paulo, Brazil) for processing. The sets were fed manually (top down) into the cleaning machine, where an adjustable, grooved, rubber roller positioned at the top of the machine transports the intestines downward into the machine toward two cleaning rollers. These rollers (turning clockwise and counter clockwise) have stainless steel ridges or ribs that scrape and remove the outer tissue layers (originally the luminal surface), mainly the mucous membrane comprising the epithelium, lamina propria, and lymphoid tissue. The submucosa, muscularis, and serosa remain to form the wall of the cleaned casing. Samples were taken after this processing step and denoted as cleaned casings. A different cleaning procedure for hog and sheep intestines is described by Koolmees and Houben (1997), which includes the complete removal of serosa and muscularis layers, leaving only the intestinal submucosa as the cleaned casing.

All sets, divided into fresh intestines (n = 40) and cleaned casings (n = 40), were individually measured, and at regular intervals covering the entire length of each set, 10 full-diameter samples (2.5 cm in length) were cut. These samples were gently flushed in saline solution (0.9% NaCl) and fixated in 10% neutral buffered formalin, making a total of 800 tissue samples.

To determine the weight reduction due to the cleaning process, a separate study was done using similar Brazilian Nelore cattle. Sets of intestines (n = 50) were

randomly selected, measured, and weighed at the first and second sampling point.

Histology

Lymphoid tissue occurs irregularly along the length of the small intestine as isolated lymphoid nodules (lymphonoduli solitarii), but tend to be most prominent in the ileum. These aggregated lymphoid nodules (lymphonoduli aggregati) are known as Peyer's patches (Junqueira and Carneiro, 1984) and are anatomically located on the convex side of the intestine opposite to the mesenteric attachment (Krölling and Grau, 1960; van Keulen et al., 1999). Complete rings of small intestine were therefore prepared for histology after fixation was complete to ensure that if any Peyer's patches were to be present in the cleaned casing, they would be detected. The subsequent histological examination, image analysis, and statistical analysis were done according to the methods described by Koolmees et al. (2004), with minor modifications. The fixated intestinal segments (horizontal cross-sections) were embedded in paraffin wax by standard methods (Drury and Wallington, 1980; Junqueira and Carneiro, 1984), and two 5-µm serial sections were cut and stained with either hematoxylin and eosin or Picrosirius red (Flint and Pickering, 1984). In total, 800 sections were prepared for each staining method from the samples collected.

First, the thickness of the intestinal wall was measured (in millimetres) using a Zeiss light microscope equipped with a projection screen (magnification of X 25). One hundred fresh intestine and 100 cleaned casing sections from each of the four slaughterhouses were measured, totalling 800 samples. In addition, a qualitative microscopic examination (magnification of X 40) was conducted of all unprocessed (fresh intestines) and processed (cleaned casings) samples to determine whether the entire mucosa including the Peyer's patches was removed. In this examination, only the presence or absence was scored (''yes'' or ''no'') using hematoxylin and eosin–stained sections.

Image analysis quantifying remaining lymphoid and neural tissue

Image analysis was done according to standard procedures for morphological measurements (Russ, 1990). Digital images of sections of the bovine intestines were taken with a uEye digital colour camera, type UI-1440-C, resolution 1,280 by 1,024 pixels (IDS Imaging Systems GmbH, Obersulm, Germany). Image analysis was carried out with a PC-based system equipped with the KS400 software, version 3.0 (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the total area of the intestinal tissue per section. The total area was measured (in square millimetres) without magnification to allow a full view of the intestinal sample.

For the image analysis of the remaining mucosa, Picrosirius red–stained sections from 5 of 10 available zones (zones 1, 3, 5, 8, and 10) were examined (magnification of X 25) from both fresh intestine and cleaned casing samples. Four squares (fields of view) per section were randomly selected and the area of viewed squares occupied by mucosa (in square micrometers) was determined. For the image analysis of the remaining lymphoid tissue, hematoxylin and eosin–stained sections from all samples containing lymphoid tissue, either before or after cleaning, were examined (magnification of X 25). The total area occupied by lymphoid tissue per section was measured (in square micrometers). Results of all measurements on remaining mucosa and lymphoid tissue were subsequently converted into percentages of the total intestinal tissue area of the section. An anti–neuron-specific enolase antibody (monoclonal; Dako, Glostrup, Denmark)

was used to stain the plexi of Meissner and Auerbach (*n* = 10), based on the methods described by Tersteeg et al. (2002). A section of brain tissue was used as a positive control. Sections were examined using a Zeiss light microscope (magnification of X 400) with a projection head and a calibrated graticule. The surface area (in square millimetres) of the plexuses was calculated from length and width measurements.

Statistical analysis

A linear mixed-effects model (Bates and Sarkar, 2006) was used with a binomial distribution for the presence for lymphoid tissue, with ‘‘animal’’ as the random effect to model the correlation between the repeated observations within an animal. The independent factors are *cleaning*; the *location* of the sample taken from the entire length of the small intestine; the *facility,* indicating the slaughterhouse; and *country,* representing local differences in cleaning technique and breed. The penalized quasi-likelihood method was used for the approximation.

A linear mixed-effects model (Pinheiro et al., 2005) has been performed with a normal distribution for lymphoid area and animal as random effect to model the correlation between the repeated observations within an animal. The lymphoid area is the dependent variable and the independent grouping variables are the cleaning method, facility, and country. The random effect was assumed to have a normal distribution. A variance model was used that allows a different variance per cleaning method. The maximum likelihood method was used for estimating the parameter effects.

For the mucosa area, the thickness of the mucosa layer was analyzed as a percentage of the thickness of the casing. The same model was used as for the lymphoid area except for the random effect for which the sample within an animal was taken.

In all analyses, the model with the smallest Akaikes Information Criterion (AIC) was selected as the best model (Pawitan, 2001). To determine a *P* value for the factors, a likelihood ratio test was performed to compare the different models. The models were fitted using the statistical program R, version 2.2.1 (R Development Core Team, 2005). Where possible, the 95% confidence intervals are given for the raw data that provide information on the accuracy of the respective findings (Diem and Lentner, 1975), but no corrections were made for repeated measurements. However, when using these data in the linear effects models, the corrections for repeated measurements were included.

Results

Thickness measurements

Table 2 summarizes the results of thickness measurements of the fresh bovine intestinal wall and of the cleaned beef casing. The results reveal that during processing, a significant decrease (one-sided Student’s *t* test: $P < 0.0001$) in thickness occurred. The standard deviation of fresh intestine thickness is high. This can be explained by the considerable differences in thickness within the intestine wall caused by the presence of valves of Kerckring (plicae circularis) (Gartner and Hiatt, 1997).

Compared with sheep and hog casings, beef casings are much thicker since they are composed of three tissue layers (submucosa, muscularis, and serosa) rather

than one (submucosa only). The muscularis and serosa are removed during the processing of sheep and hog casings (Ockerman and Hansen, 2000).

Table 2: Thickness measurements intestinal wall, before and after processing

Samples	Sections Examined (*n*)	Thickness (mm)[a]	SD
Fresh intestines	400	3.01 (2.92 - 3.1)[a]	0.96
Cleaned casings	400	1.15 (1.12 – 1.18)[a]	0.28

a) 95% confidence interval for the mean

Weight reduction after cleaning

The lengths and respective weights of 50 sets of intestines were determined at the first and second sampling point and recorded, representing fresh intestines and cleaned casings (Table 3). On average, the bovine small intestinal tract yields 35 ± 4 m available for casing production. With an average weight of 3,900 g for the fresh intestines and 2,264 g for the cleaned casings, a weight reduction of 42% was achieved, mainly due to the removal of the mucosa (Table 4).

Table 3: Average weights and length of 50 sets of intestines before (fresh intestines) and after cleaning (cleaned casings)

Average length[a]	Average weight (g)[b]	SD	Relative weight (g/m)[b]	SD
Fresh intestines	3,900 [3,658 – 4,142][a]	874	110.7 [105.6 – 115.8][a]	18.4
Cleaned casings	2,264 [2,099 – 2,429][a]	596	64.0 [60.4 – 67.6][a]	13.0

a) 35 m ± 4 m, *n* = 50, b) 95% confidence interval for the mean

Table 4: Mucosa tissue present as % of total thickness

Samples	Sections Examined (*n*)	Total mucosa area (%)[a]	SD
Fresh intestines	80	44.65 (43.3 – 46)	6.16
Cleaned casings	400	4.39 (4.23 – 4.55)	1.6

a) 95% confidence interval for the mean

Mucosa

In the qualitative analysis, samples were scored on the presence of mucosa, before and after cleaning. As expected, all samples from fresh intestines (*n* =400) contained mucosa. However, all samples from cleaned casings (*n* = 400) also contained residual amounts of mucosa. Regarding the quantitative analysis, the lowest AIC was found when only the independent factor *cleaning* was taken into account. This means that the other factors (location, facility, and country) did not contribute to the differences found between cleaned casings and fresh intestines. There was a significant ($P < 0.0001$) reduction of 90% in mucosa tissue present in cleaned casings compared with fresh intestine (Table 4).

Lymphoid tissue

In the qualitative analysis, samples from fresh intestines ($n < 400$) and cleaned casings ($n < 400$) were scored for the presence of lymphoid tissue. In contrast to the presence of mucosa in all samples, lymphoid tissue was only found to be

present in 60 (15% positive score) of the 400 fresh intestinal samples and 40 (10% positive score) of the 400 cleaned casing samples (Table 5).

Table 5: Number of samples[a] with lymphoid tissue present

Samples	Sections Examined (*n*)	Brazil 1	Brazil 2	Uruguay 1	Uruguay 2	Total
Fresh intestines	400	18[a]	17	12	13	60
Cleaned casings	400	12	9	12	7	40

a) Number of samples found positive in each facility

There was a significant difference ($P < 0.05$, odds ratio of 1.60, 95% confidence interval of 1.01 to 2.53) in the number of samples containing lymphoid tissue when comparing fresh intestines ($n = 60$) with cleaned casings ($n = 40$). As only cleaned casings will be used for sausage production, for risk assessment purposes, account should be taken of the 1:10 incidence ratio for the presence of lymphoid tissue.

The lymphoid tissue observed consisted of Peyer's patches (aggregated lymphoid nodules or lymphonoduli aggregati), remains thereof, and isolated lymphoid nodules (lymphonoduli solitarii). These different forms of lymphoid tissue also account for the high standard deviations calculated in the different quantitative analyses.

The quantitative analysis of lymphoid tissue reduction was done only in those samples that were scored positive in the qualitative analysis (see Table 5). Contrary to the mucosa analysis, the lowest AIC was found when the independent factors *cleaning* and *country* were taken into account for the lymphoid tissue. As the *location* from where the samples were taken along the entire length of the small intestine had no influence on the results, this indicated that after cleaning a homogenous distribution of lymphoid tissue in the duodenum and jejunum occurred.

On average, the total lymphoid area of the Uruguayan fresh intestines was 8.6 mm^2 and the cleaned casings 4.5 mm^2. The cleaning effect was significant ($P = 0.00$), with a 48% reduction in lymphoid tissue. The Brazilian fresh intestines had on average a total lymphoid area of 6.7 mm^2 and the cleaned casings an area of 2.6 mm^2 ($P = 0.00$, 61% reduction). A significant difference ($P = 0.0006$) existed between the total lymphoid areas of the Uruguayan and Brazilian samples, between either the fresh intestines or the cleaned casings. However, based on a likelihood ratio test, the interaction effect between cleaning method and country was not present in the final model, as the difference between both countries in cleaning efficiency was not significant ($P = 0.508$).

A second statistical analysis was done in order to determine the area of lymphoid tissue as a percentage of total thickness. As with the previous best-fit analysis, the lowest AIC was found when the independent factors - *cleaning* and *country* - were taken into account. The second analysis revealed a significant estimated difference of 2.32% ($P < 0.001$) in total lymphoid tissue area percentage, which confirmed the quantitative reduction of lymphoid tissue after cleaning (results in Table 6). The second analysis also confirmed the absence of interaction between cleaning method and country in the final model, as the likelihood ratio test was not significant ($P = 0.169$).

Table 6: Lymphoid tissue present as % of total thickness

Samples	Sections Examined (*n*)	total lymphoid area (%)[a]	SD
Fresh intestines	60	8.21 6.54 – 9.88	6.61
Cleaned casings	40	5.24 3.88 – 6.6	4.37

a) 95% confidence interval for the mean

Neural tissue

To illustrate the presence of neural tissue after cleaning, a quantitative analysis on its presence in samples from fresh intestines was done. Several samples (*n* = 10) were used that had scored positive on the presence of lymphoid tissue in the previously described qualitative analysis. The plexi of Meissner and Auerbach, stained with the anti–neuron-specific enolase antibody, were estimated to cover an area of 0.14% (data not shown). Samples from cleaned casings were not included in this study, as the cleaning process will not remove any of the neural plexi.

Discussion

Several different cleaning procedures are in place within the casing industry for the processing of bovine intestines into beef casings. However, the differences in these procedures relate mostly to the level of automation, whereas the general principle of cleaning remains the same throughout. A pilot study was done by Koolmees in 1998 on the histology of beef casings, using a fully automated process (H&H automatic beef casing line, type 60-120RD4-5 Holdijk & Haamberg GmbH, Gronau, Germany). Preliminary results found in this study were compared with the current quantitative analyses, indicating a similar level of cleaning efficacy. Therefore, as all cleaning procedures are based on the same cleaning technique, and there was no *country* effect on the quantitative results found in the current study, it can be inferred that all cleaning procedures will lead to a similar quantitative reduction of mucosa and lymphoid tissue. As shown, beef casings retain some mucosa and lymphoid tissue after the cleaning process, and all layers of the intestinal wall can be distinguished (Figure 1).

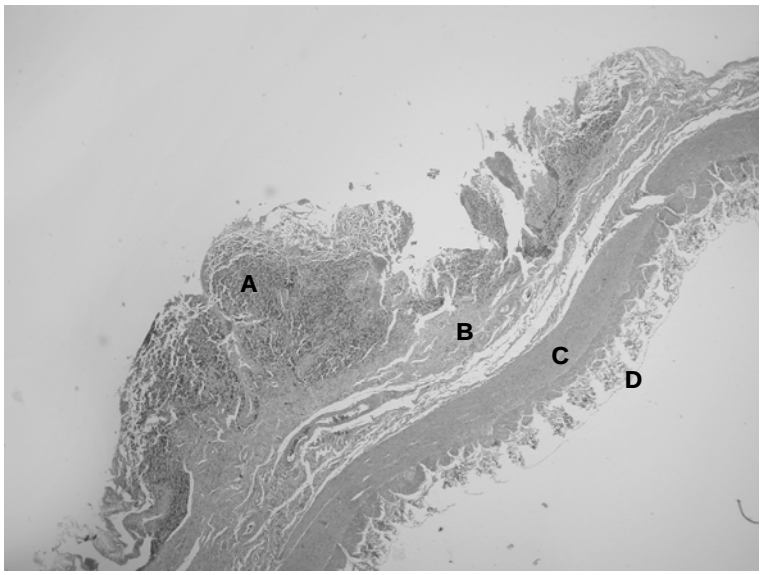

Figure 1: Cleaned beef casing (heamtoxylin and eosin, X 25 magnification) with remains of Peyer's patches (A) embedded in mucosa, submucosa (B), musculary layers (C) and serosa (D)

This also means that no neural tissue is removed, leaving the plexi of Meissner (embedded in the submucosa) and Auerbach (between the circular and longitudinal muscle layer) intact. Previous reference to histological textbooks (Krölling and Grau, 1960; Gartner and Hiatt, 1997) shows lymphoid tissue to occur irregularly along the length of the small intestine as isolated lymphoid nodules (lymphonoduli solitarii) or as Peyer's patches (lymphonoduli aggregati). However, results indicate that due to the cleaning process, which removes Peyer's patches completely or partially, a more homogenous distribution pattern of lymphoid tissue occurs, based on these remnants and remaining isolated lymphoid nodules. These findings are substantiated by the fact that in the quantitative analysis of lymphoid tissue, the location from where the sample was taken along the entire length of the small intestine was without influence on the results. This is of great importance, as it will allow for a 1:10 incidence ratio on the presence of lymphoid tissue in a subsequent risk assessment, which can only be assumed when a homogenous distribution exists.

The presence of lymphoid tissue and myenteric plexi (Figure 2) in cleaned beef casings does not implicate an inherent risk in transferring BSE. Sheep casings are considered to have a negligible BSE infectivity risk as Peyer's patches are removed completely after cleaning. From the neural tissue, only the plexus of Meissner will remain, as it is part of the submucosa that constitutes sheep casings (Koolmees et al., 2004).

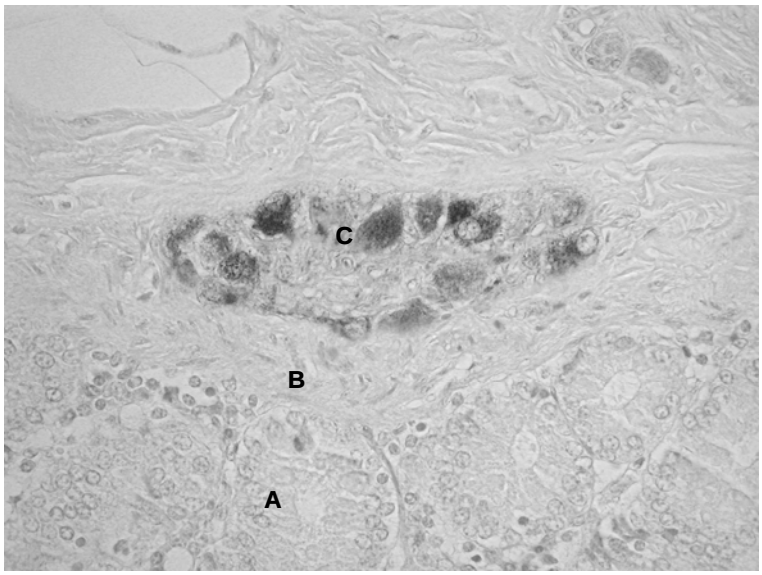

Figure 2: Fresh intestine (anti-neuron-specific enolase antibody, counterstaining with Mayer's haemotoxylin, X 400 magnification). Mucosal villi (A), submucosa (B) and plexus of Meissner (C)

Contrary to the pathogenesis in sheep and mice, BSE infectivity in cattle has, up to now, an exclusive intraneuronal spread from the intestinal tract to the central nervous system (Buschmann and Groschup, 2005; Hoffmann et al., 2007). Only in some of the clinically affected, experimentally induced and naturally occurring cases of BSE could sparse immunostaining of neurons in the myenteric plexi and disease-specific prion protein be detected in the Peyer's patches of the distal ileum (Terry et al., 2003). In cattle incubating BSE, disease-specific prion protein was only detected in the Peyer's patches of the distal ileum, not in the myenteric plexi of the distal ileum or elsewhere (Hoffmann et al., 2007). Therefore, a negligible BSE risk can be assumed, as the distal ileum is removed completely prior to the processing of bovine intestines into beef casings, and the duodenum and jejunum are regarded as free of BSE (Wells et al., 1994; Terry et al., 2003; Buschmann and Groschup, 2005; Hoffman et al., 2007).

Using a similar argumentation based on existing literature, the U.S. Food Safety and Inspection Service (FSIS) amended in October 2005 its rule ''Prohibition of

the Use of Specified Risk Materials for Human Food and Requirements for the Disposition of Non-Ambulatory Cattle'' (US Food Safety and Inspection Service, 2005). This amendment re-allowed the use of beef casings for the production of sausages originating from countries with a GBR II categorization, indicating a controlled BSE risk according to World Organisation for Animal Health standards (World Organisation for Animal Health, 2007). A critical point in the FSIS amendment and World Organisation for Animal Health Terrestrial Animal Health Code is the complete removal of the distal ileum from bovine intestines and prohibited use in the preparation of foodstuffs. This requirement is already fulfilled, as the ileum is unfit for casing production due to an aberrant shape and texture. It is therefore a standard operating procedure to remove and destroy the ileum prior to the processing of bovine intestines into beef casings (ENSCA, 2006). Results indicate that approximately 50% of the total weight and thickness of the intestine was removed during the cleaning process (Tables 2 and 3). Mucosa is removed for 90% but remains present in all samples tested. Lymphoid tissue was either not present in 85% of the samples from fresh intestines or was removed completely in 90% of the samples from cleaned casings. In the samples from cleaned casings, 5.24% of total lymphoid area remained as percentage of the total thickness of the cleaned casing (Table 6). Assuming that the relative contribution of each specific tissue layer to the total weight is equal, a direct correlation is possible between the thicknesses of the relevant layers of the small intestine and their respective mass (Table 7). Taking into account the 1:10 incidence ratio of lymphoid tissue being present, it leads to a calculated area percentage of 0.52 for lymphoid tissue. Based on these data, it can be estimated that 340 mg of lymphoid tissue and 90 mg of neural tissue remain per meter of cleaned casing.

Table 7: Calculated weights of the different layers of cleaned casings

Lymphoid tissue present	As area %	Approx. weight of layers (g/m)
Mucosa	4.39	2.81
Lymphoid tissue	0.52	0.34
Neural tissue	0.14	0.09
Combined layer[a]	94.95	60.76
Total	100	64.0

a) consisting of submucosa, muscularis and serosa

From an average length of 35 m of processed beef casings, 30 m is used for sausage production, with a stuffing capacity of 25 kg/30 m. Sixty sausages can be produced from each cut, as one standard sausage made in beef casings measures 50 cm and weighs approximately 400 g. If a meal consisted of half a sausage (200 g), then the casing envelope would be 25 cm long (ENSCA, 2006). According to the presented data, consumption of the entire 25 cm would result in the ingestions of approximately 0.09 g of lymphoid tissue and 0.02 g of neural tissue.

The results from this study clearly illustrate the limited presence of lymphoid tissue in cleaned beef casings, by incidence and calculated amount. As BSE infectivity remains unconfirmed in both lymphoid and neural tissue present in the intestinal tract used as beef casings and the final exposure to the consumer is extremely limited, it can be suggested that beef casings carry a negligible risk in transferring BSE. Additional research using beef casings originating from BSE-infected cattle could further corroborate these findings.

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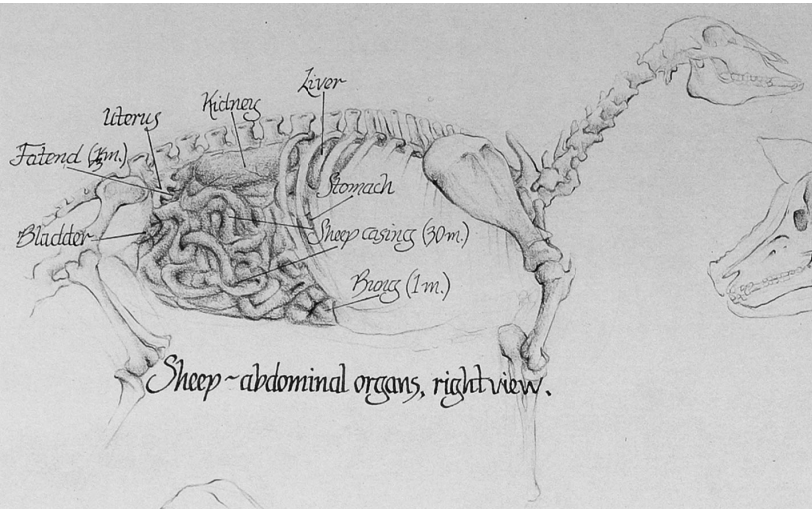
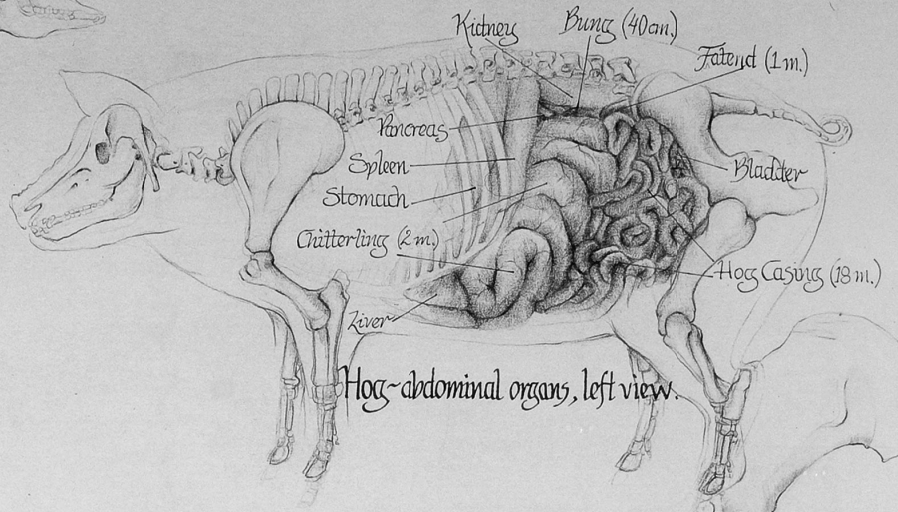
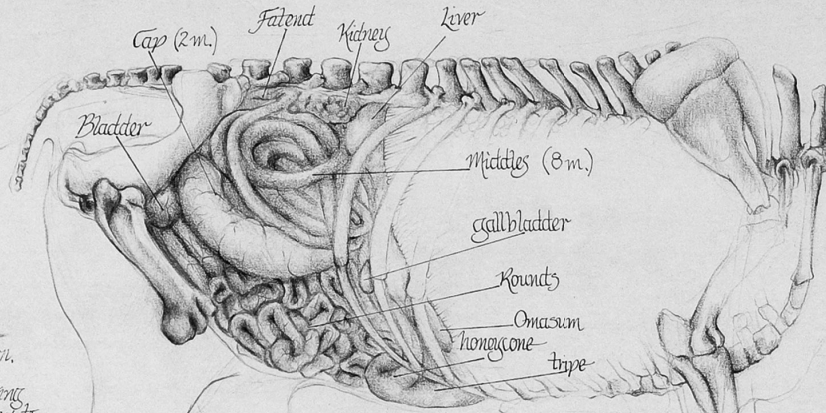


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 5

Removal of foot-and-mouth disease virus infectivity in salted natural sausage casings by minor adaptation of standardized industrial procedures

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Based on:
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Abstract

Intestines are used for the production of natural casings as edible sausage containers. Derived from animals (pigs and sheep) experimentally infected with FMDV (initial dosage $10^{7.3}$ pfu / ml, strain $O_{1Kaufbeuren}$), these casings were treated with salt (NaCl) or phosphate supplemented salt and the residual FMDV titres measured. After storage at about 20 °C, no remaining infectivity was found after either treatment, whereas casings stored at 4 °C still contained infectivity. Storage of salted casings at about 20 °C for 30 days is already part of the Standard Operating Procedures (included in HACCP) of the international casing industry and can therefore be considered as a protective measure for the international trade in natural sausage casings.

Introduction

Foot-and-mouth disease (FMD) belongs to the most economically important contagious diseases of cloven-hoofed animals. The disease is notifiable to the OIE, the World Organisation for Animal Health and various measures are in place to prevent its spreading. In order to establish a scientific basis for measures to prevent the spread of FMD virus via food products from infected animals, several studies have been done in order to determine its survival in various products. For example, Panina et al. (1989) showed that lactic acid formation in fermented sausages led to complete loss of any FMD virus infectivity. However, practically all of these studies focused on the potential survival of FMD virus in fresh meat or meat products, but not in the casings surrounding these products. Only McKercher et al. (1978, 1980) mentioned that residual infective FMDV remains in untreated processed casings for as long as 250 days. Unfortunately they provide neither reference to the original studies on the casings involved, nor information on processing and storage of these casings (temperature, pH, salting).
In the absence of specific data, risk assessments have to be extrapolated to products or diseases which have not been investigated - which may lead to false conclusions. This may happen due to differences in the pathogenesis of diseases (Alexandersen et al., 2003) as well as in the organ distribution of infectious agents and their ability to survive in different food products. As a result, either insufficient reduction of risks may occur or unnecessary trade restrictions are implemented. To determine the efficacy of certain methods available for the inactivation of micro-organisms, only the product itself can serve as its own model to make an objective assessment (Park and Beuchat, 2003; Wijnker et al., 2006).

Table 1: Virus titres in specific tissues of experimentally infected sheep[1]

	Sheep nr 570	Sheep nr 527	Sheep nr 526
	Killed 2 days post infection (p.i.)	Killed 3 days p.i.	Killed 4 days p.i.
Blood	-[a]	4.75[b]	0
Tonsils	3.2	4.0	3.25
Lymph nodes small intestines	3.5	2.5	2.5
Lymph nodes large intestines	3.8	2.5	2.5
Liver	0	2.5	2.5
Lung	0	3.0	2.5
Spleen	0	2.5	2.5
Kidneys	0	3.0	2.5
Heart muscle	0	0	0
Tongue surface (lesion)	0	4.75	0
Tongue muscle	0	0	2.5
Stomach	0	1.75	2.5
Small intest. Emptied	2.2	2.5	0
Small intest. Washed	0	2.5	2.0
Small intest. Scraped	**2.5**	**2.5**	**2.5**
Large intest. Emptied	3.2	2.75	2.5
Large intest. Washed	3.8	2.5	2.5

a) Not done; b) Log $TCID_{50}$/ 1.0 ml

[1] Table reprinted with consent of publisher (Böhm and Krebs, 1974)

In 1974 Böhm and Krebs not only reported different FMD virus titres in specific tissues of experimentally infected sheep (Table 1), but they also confirmed the efficacy of a 5-minute treatment with 0.5% citric acid on infected sheep casings to inactivate the FMD virus. Although this was also mentioned by McKercher et al. in 1978, no other studies on FMD virus inactivation in casings are presently known. The objective of this study is to determine the efficacy of reducing FMD virus titres to safe levels in processed casings. To this effect casings are treated with salt or the proposed phosphate supplemented salt and stored under conditions which closely resemble those standardized by the casing industry. A storage period of 30 days for salted casings is also one of the treatments described in Commission Decision 2004/414/EC as regards the animal health conditions and veterinary certification for animal casings and the EC Council Directive 2003/85 on community measures for the control of foot-and-mouth disease. The results of the study will indicate whether EC legislation and industry Standard Operating Procedures should be amended as a protective measure against the spread of FMDV via casings.

Materials and methods

In previous studies citric and phosphate supplements with NaCl were regarded as suitable additives in casings. (Bakker et al. 1999; Ockerman and Hansen, 2000). As citric acid was already confirmed as an effective agent against FMDV in naturally infected casings (Böhm and Krebs, 1974), FMDV inactivation at low pH was not studied again. The FMD virus infectivity study was done at the Friedrich Loeffler Institute (FLI, Insel Riems), the German National Reference Laboratory for FMDV.

Inactivation of cell culture virus

A cell culture supernatant of $O_{1Kaufbeuren}$ with an initial titre of 10^8 $TCID_{50}$ per ml was adjusted to a pH of 10 with phosphate supplemented salt to determine possible inactivation of the FMD virus. Aliquots were neutralized after 30, 60 and 120 minutes to a pH of approx. 7.5 and tested for remaining infectivity as described below.

Animals

In total ten fattening pigs (approx. 110 kg, 6 months) and ten sheep (approx. 35 kg, 3.5 months) were purchased locally and used for this study. All animals were not vaccinated for FMD, came from disease-free locations and were in perfect health.

Virus strain and initial dosage

Preceding the main experiment, a pilot study with two pigs and two sheep using virus strain $O_{1Kaufbeuren}$ and two pigs and two sheep using virus strain $A_{Iran2/97}$ was carried out. Both strains are known for their relevance in previous FMD outbreaks and were available at the Friedrich Loeffler Institute. Virus strain $O_{1Kaufbeuren}$ was chosen for the main experiment because only infection with this strain resulted in titres and clinical signs that warranted further experiments (data not shown). For the main experiment six pigs and six sheep received an initial dosage of $10^{7.3}$ pfu / ml of $O_{1Kaufbeuren}$ by simultaneous intranasal infection and injection into the bulb of the heel. The initial virus titre dosage was determined according to the method described under *Analysis of intestinal samples*.

Phosphate supplemented salt
The phosphate supplemented salt contained 86.5% NaCl, 10.7%, Na_2HPO_4 and 2.8%, Na_3PO_4 (wt/wt/wt).

Intestinal samples
Animals were slaughtered after the onset of clinical signs. The animals were electrically stunned and after exsanguination, the abdominal cavity was opened and the intestinal tract removed. The intestines were processed manually into casings by the institute's butcher. After pulling the intestines from the mesentery the small and large intestines were separated for further processing. The manure was squeezed out and the intestines were then scraped using a bent stick, removing the mucosa, outer serosa and musculary layers, leaving only the submucosa as final layer (Koolmees et al., 2004). Representative samples from both small and large intestines were taken and assigned to one of four treatment groups, which were treated with either salt or phosphate supplemented salt at 4 °C or room temperature of approximately 20 °C.
Samples were dry-salted and stored for 30 days according to the respective treatment group. Prior to further virus titre analysis, the samples were rinsed in water to remove any attached salt or phosphate supplemented salt.

Analysis of intestinal samples
For virus isolation, a protocol derived from the FLI diagnostic standard protocol and the O.I.E. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (5^{th} edition, 2004) was used. Per animal (n = 12) and respective treatment group (8 in total), pooled samples were made from 25 cm of intestines per sample. In short, intestine samples were cut into postal stamp-size pieces and homogenised in cell culture medium. The homogenate was treated with chloroform to inactivate bacteria and enveloped viruses and centrifuged to remove cell debris. The supernatant was put onto a monolayer of BHK21-CT cells. Negative cultures were passaged twice and positive results (cytopathogenic effect or CPE) were checked for specificity by antigen-ELISA.
In order to generate quantitative data (plaque forming units, pfu per volume), part of the samples were also investigated in a plaque test performed in dishes with cells suspended in agar as described by Moss and Haas (1999). For the cell suspension plaque test and subsequent virus titre analysis, BHK21-CT cells were also used.

Statistical analysis
For statistical analysis a McNemar's test (Petrie and Watson, 2000) was used to compare paired observations based on the presence or absence of virus before and after treatment with the phosphate supplemented salt (virus present yes or no). The null hypothesis, being there is no difference between virus presence before or after treatment, will be rejected when $P<0.05$. A Chi-squared distribution with one degree of freedom will determine whether the null hypothesis can be rejected.

Results

Cell culture virus

The results of the inactivation of cell culture virus at pH 10 are shown in figure 1. A virus titre reduction of 3 $\log_{10}$ $TCID_{50}$ / 0.1 ml is achieved after 120 minutes, indicating a potential virus-inactivating effect of the phosphate supplemented salt used.

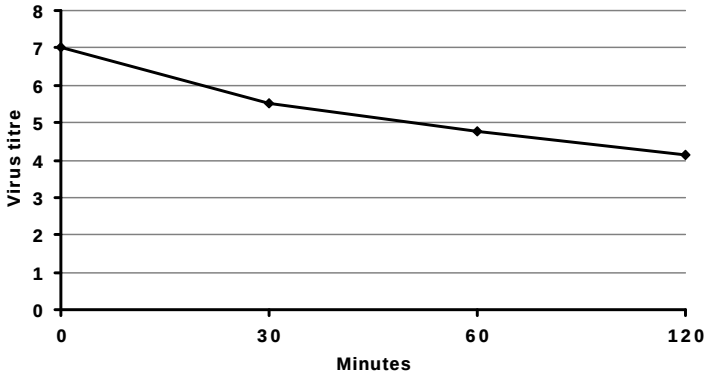

Figure 1: Cell culture virus titre reduction at pH 10

Animal infection experiments

In table 2 an overview is given of the noted changes in body temperature and determined virus titre (in $\log_{10}$ pfu / ml) on the day of slaughter of the respective animal.

Table 2: Temperature and virus titre at slaughter

	Day 0	Day 1[a]	Day 2	Day 3	Day 4	Day 5	Serum[c]	Large intestine[c]	Small intestine[c]
Pigs									
No.1	38.3	38.4	38.1	39.3	39.2	39.0[b]	- -	- -	- -
No.2	38.2	38.7	41.2[b]				Pos.	2.56	Trace
No.3	38.4	38.6	39.0	38.9	41.2[b]		Pos.	0.95	- -
No.4	38.4	38.9	39.2	39.8[b]			Pos.	1.35	- -
No.5	38.2	38.5	38.9	39.1	40.9[b]		Pos.	0.4	- -
No.6	38.5	39.3	39.4	40.0[b]			Pos.	- -	0.36
Sheep									
No.1	39.1	40.2	40.1	40.4[b]			Pos.	1.0	- -
No.2	39.2	40.4	40.6	40.2[b]			Pos.	1.0	0.36
No.3	39.4	40.6	40.7[b]				Pos.	3.0	2.2
No.4	39.0	40.1	39.8	39.6	39.7	39.9[b]	- -	- -	- -
No.5	39.2	40.7	40.5	40.4	40.0[b]		- -	0.4	- -
No.6	39.0	40.3	40.6	40.2	39.6	39.7[b]	- -	- -	- -

a) Day after infection, body temperature in °C; b) Day of slaughter;
c) Virus titre in $\log_{10}$ pfu / ml

No virus could be isolated in any sample from the large or small intestines (*n* = 12) stored for 30 days at room temperature and treated with either salt or phosphate supplemented salt. However, samples of five animals (*n* = 12) still contained infectivity after storage at 4 °C (Table 3).

In order to determine the efficacy of the method, all animals with a positive titre in serum, large intestines or small intestines at the moment of slaughter are regarded as being infectious and therefore a potential risk of FMD virus transmission. In nine out of twelve animals FMDV infectivity was found before the treatment, whereas no infectivity was found after storage at room temperature.

Table 3: FMD virus isolation after 30 days of treatment by various methods

	Large intestine				**Small intestine**			
	20 °C		4 °C		20 °C		4 °C	
	Salt	P + salt[a]	Salt	P + salt	Salt	P + salt	Salt	P + salt
Pigs								
No.1	-	-	-	-	-	-	-	-
No.2	-	-	-	-	-	-	-	-
No.3	-	-	-	-	-	-	-	-
No.4	-	-	-	-	-	-	-	-
No.5	-	-	Pos.	-	-	-	Pos.	-
No.6	-	-	-	-	-	-	-	-
Sheep								
No.1	-	-	Pos.	Pos.	-	-	-	-
No.2	-	-	Pos.	-	-	-	Pos.	-
No.3	-	-	-	Pos.	-	-	Pos.	-
No.4	-	-	-	-	-	-	-	-
No.5	-	-	-	Pos.	-	-	-	-
No.6	-	-	-	-	-	-	-	-

a) P + salt: phosphate supplemented salt

Using the McNemar's test, the calculated *P*-value is between 0.001 and 0.01. Therefore, the finding that no FMDV infectivity could be detected in salted casings stored at room temperature for a minimum period of 30 days is significant. However, based on the results found, no difference could be made between the salt treatment and the phosphate supplemented salt.
Virus titres could be found in the samples stored at 4 °C, although they could not be quantified. Using the McNemar's test no difference could be found between the presence before or after treatment.

Discussion

The animal infection experiments show that intestines originating from infected animals can be effectively treated to remove all FMDV infectivity. The McNemar's test indicates that the probability (*P*-value) of not finding a positive sample while the treatment is not effective is less than 1%. The statistical power of this study, being the chance of detecting the significant difference before and after treatment, was more than 95%. Therefore it can be stated that the storage of casings treated with salt or phosphate supplemented salt for 30 days at room temperature is sufficient to remove FMDV infectivity. The results support that, in principle, the treatment laid down in Commission Decision 2004/414/EC and the EU Council Directive 2003/85 is effective. However, our results suggest that the storage temperature is of critical importance.
In the review article by Alexandersen et al. (2003) on the pathogenesis and diagnosis of FMDV, a clear overview is presented of the primary sites of infection and secondary sites of replication. Based on the information presented it can be suggested that the FMD virus will primarily replicate in tissues of embryonic ectodermal origin (Noden and De Lahunta, 1985), mainly oral cavity and skin. Based on the endodermal origin of the intestinal tract it can therefore be suggested that the virus titre found in cleaned casings is most likely based on the minimal amount of blood remaining after processing. This is supported by

Oleksiewicz et al. (2001) on the quantification of FMDV in diverse porcine tissues. In this study the following observations were made: Tissue titres of heart (mesoderm), kidney (endoderm), liver (endoderm) and spleen (mesoderm) were remarkably uniform (10^2-10^3 $TCID_{50}$) and distinctly lower than the peak viraemia titre of 10^6-10^7 $TCID_{50}$, found 2 days post-infection. Both liver and spleen are likely to be involved in the active clearance of viraemia by phagocytosis. Therefore the FMDV concentrations found in these tissues probably represent the absolute maximum which could be attained by serum contribution and sequestration of virus. As a result, higher virus concentrations are more indicative of active FMDV replication and were found in tissues of ectodermal origin. This may account for the consequently low FMD virus titres found in cleaned casings, not only in our animal experiments (max. $10^{3.0}$ pfu / ml) but also in the original study by Böhm and Krebs (1974, Table 1).
Additionally, Alexandersen et al. (2003) reported on the survivability of FMDV. Of great interest is the biphasic kinetic curve for the decay of FMDV infectivity. As shown in our results of the cell culture virus titre reduction at pH 10, an initial steep decay is followed by a prolonged, shallow tail of residual infectivity. In the main animal infection experiments the temperature during storage is kept at around 20 °C. According to Bakker et al. (1999), storage at lower temperatures does not significantly improve the quality of the salted casings and is therefore not included in the standard procedures of the industry. Our results showed no positive samples after 30 days for the salted casing samples. This indicates that the time set for minimal storage is sufficient to have any residual infectivity removed completely when stored at around 20 °C.
Much of the information on virus susceptibility by various routes to different animals is presented as 50% Tissue Culture Infective Dose ($TCID_{50}$). Due to the calculation of titres as pfu / ml, a conversion into $TCID_{50}$ is necessary in order to make a comparison possible. The following equation (Horzinek, 1985) being that the ID_{50} equals about 0.7 pfu or 1 pfu equals about 1.4 ID_{50} was deemed sufficient for this purpose.
Sellers (1971) reported on the experimental infection dose by oral route being about 10^4-10^5 $TCID_{50}$ for pigs and 10^5-10^6 $TCID_{50}$ for ruminants (see also review Alexandersen, 2003). The highest titre found in this study for untreated intestines was 1000 pfu or about 1400 $TCID_{50}$ per ml of homogenate. When fed to susceptible animals, several grams of untreated intestines may thus lead to an oral infection. Be that as it may, intestines processed into natural sausage casings are only traded and imported into the EU after salting and storage of at least 30 days, therefore meeting the requirements as laid down in current EU legislation. Although blanching and drying are also permitted as alternative methods, these are not commonly used by the industry.
When reviewing studies done in the late sixties on the survivability of FMD virus in animal products, ample arguments can already be found to support salt-curing of casings as a valid virus-inactivating method. However, these studies never focussed on intestines in particular and did not take into account the standard processing (cleaning and salting) and storage conditions (minimum period 30 days, room temperature) of casings. Heidelbaugh and Graves (1968) showed a 5 log_{10} virus titre reduction in lymph nodes in salt-cured beef stored for 33 days at 3.3 °C. Although the rate of reduction was deemed too slow for commercial use in beef products, the same rate will be more than sufficient for salted casings stored at room temperature for 30 days. Savi et al. (1962) reported the presence of FMD in uncleaned and unsalted intestines after 120 days, stored at temperatures between 2-4 °C, or at -30 °C. In contrast, Cottral (1969) reported

on a 6-day survival of FMD virus in intestines stored at 1-7 °C. Cottral also proposes to use a high salt concentration with an alkaline pH as preservative with FMDV inactivating properties. This is further substantiated by Vande Woude (1967) and Sellers (1968) showing the possibility of a 3 $\log_{10}$ virus titre reduction (initial FMD virus titre $10^{5.5}$-$10^{7.5}$ pfu / ml) at a pH of 10.75, over a period of 30 minutes. The residual infectivity found in our study in casings stored at 4 °C for 30 days under alkaline conditions may be due to a protective effect of proteins and lipids in salted casings.
Although specific reference is made (McKercher et al., 1978; Blackwell, 1984; Farez and Morley, 1997) to the residual presence of FMDV in cleaned hog casings, no experimental details are available on how these results were obtained. This unclear situation may explain the high risk classification of casings in several recent risk assessments (e.g. AQIS, 1999; EFSA, 2006).

Conclusion

Regarding the objectives of this study the following conclusions can be made: The storage of casings treated with either salt or phosphate supplemented salt at about 20 °C for 30 days can be deemed sufficiently effective to inactivate a possible contamination with FMDV. In general, these storage conditions of salted casings are already part of the SOPs by the international casing industry due to logistic reasons and quality assurance. However, some companies may prefer storage at lower temperatures and in this case the proposed storage requirements can be implemented to remove a possible contamination with FMDV. A recommendation could therefore be made to have the temperature requirement entered together with the minimum storage period into specific legislation or measures to prevent the spread of FMDV.
As shown by the cited literature and proper reference of original studies, the potential risk of FMDV transmission through salted natural sausage casings can be reduced to an acceptable minimum without major changes to the common practises of the casing industry. The results of this study show that the proposed phosphate supplemented salt does not significantly enhance the inactivation of FMDV infectivity. Nevertheless, its use may become appropriate when the possible spread of other important viral diseases, such as classical swine fever, through casings is to be prevented.

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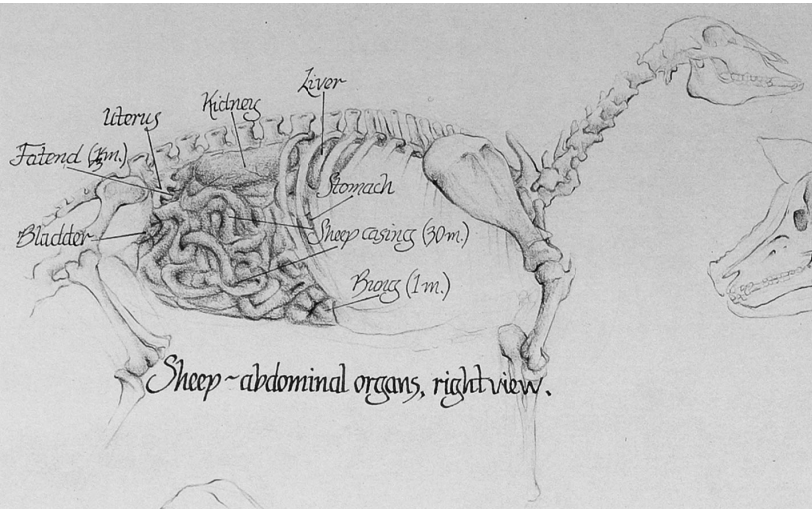
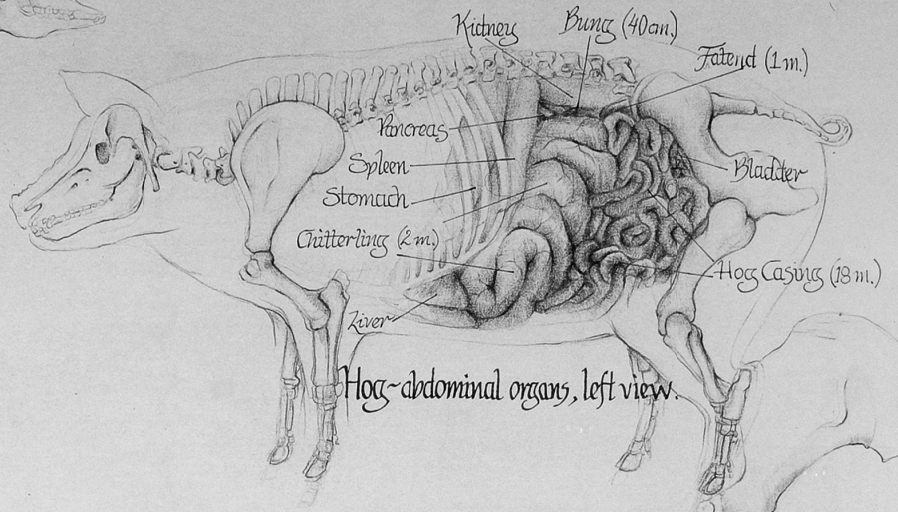
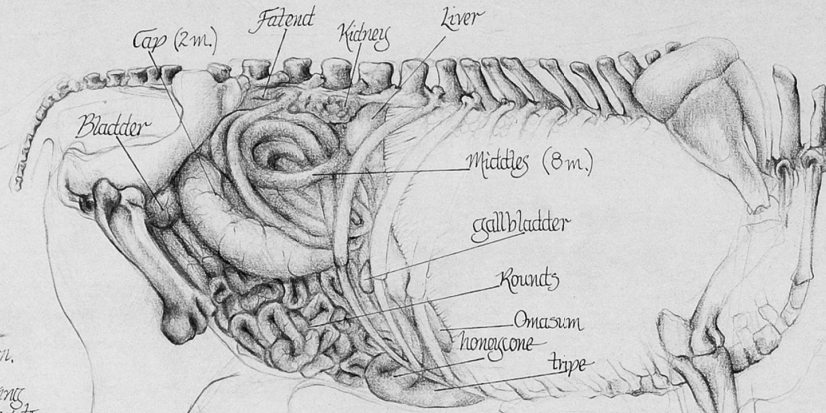


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 6

Inactivation of classical swine fever virus in porcine casing preserved in salt

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Abstract

Pig intestines used for the production of natural sausage casings may carry classical swine fever (CSF) virus. Feeding pigs with human food waste that contains pig casings may then spread the virus to CSF-free animals. Casings derived from a pig experimentally infected with CSF by dosing with 10^6 tissue culture infectious doses ($TCID_{50}$) of the highly virulent CSF virus strain “Koslov”, were treated with phosphate supplemented or citrate supplemented NaCl, instead of with NaCl alone, which is the standard preservation treatment for casings. Treated casings were stored for 30 days at either 4 °C or 20 °C. After storage the casings were fed to 16 susceptible pigs. CSF infection was confirmed in the four animals that had been fed casings treated with citrate supplemented salt and stored at 4 °C. All other animals remained healthy. It is therefore possible to avoid the inadvertent spread of CSF virus via porcine sausage casings by treating casings with phosphate supplemented salt and storing them for 30 days at temperatures over 4 °C.

Introduction

Classical swine fever (CSF) is a highly contagious disease in pigs that can cause severe economic losses. CSF is notifiable to the World Organisation for Animal Health (www.OIE.int) and various measures are in place to prevent its spread. Although animals found to have the disease are destroyed it is possible for CSF virus (CSFV) to be spread via human food products derived from subclinically infected swine, when food waste is -illegally- fed to CSF-free domestic or feral pigs (Fritzemeier et al., 2000; Wooldridge et al., 2006).
Most studies of the survival of CSFV in foods have involved meat products without consideration of the casings that are used (Panina et al., 1992; Edwards, 2000). Only two studies included an investigation of CSFV infectivity in processed casings (Helwig & Keast, 1966; McKercher et al., 1980). However, the data from these studies do not allow for a risk assessment of CSFV infection from processed casings.
To inactivate CSFV infectivity in casings which are subsequently used for the production of sausages, an antiviral agent that can be used with casings is required. In a study done by Depner et al. (1992), CSFV survivability was shown to be dependent on pH and temperature. At any pH, the half-lives of several CSFV strains were markedly shorter at 21 or 37 °C than at 4 °C.
Böhm and Krebs (1974) showed that foot-and-mouth disease virus (FMDV) can be readily inactivated by using 0.5% citric acid, whereas Wijnker et al. (2007) showed that FMDV in infected casings can be inactivated by treatment with either salt or phosphate supplemented salt during 30 days of storage at 20 °C.
Studies on the use of citric, lactic and phosphate supplements with NaCl for the processing of casings showed a clear reduction of the bacterial load by all three additives (Bakker et al., 1999), with phosphate being the preferred supplement, because casings prepared using it handled better during the stuffing process and had improved tenderness (Verkleij & Keizer, 2003; Houben et al., 2005; Nakae et al., 2008).
The objective of this study was to determine the efficacies of phosphate or citrate supplemented salt for removing CSFV infectivity from casings, and so to identify a possible precautionary treatment for the prevention of CSFV spread via porcine sausage casings.

Materials and methods

All chemicals used in these experiments were laboratory grade and supplied by Sigma-Aldrich Chemie GmbH (Munich, Germany) unless otherwise specified.

Animals

A fattening pig, about 5 months old and weighing approximately 90 kg, and 16 weaned piglets, that each weighed approximately 15 kg were purchased locally. The animals had not been vaccinated for CSF, came from disease-free facilities and were in good health. The fattening pig was used for to obtain infectious intestines and the 16 piglets were used for the oral infection experiment. The piglets were divided into eight pairs of animals and each pair was isolated from all the other groups.

Infection experiments and processing of infectious material

The pig was injected intramuscularly with 10 ml of a suspension of the highly virulent "Koslov" strain of CSFV. The inoculum contained 10^6 tissue culture infectious doses ($TCID_{50}$). Six days later, after the onset of fever, the animal was electrically stunned and exsanguinated. The abdominal cavity of the carcass was opened and the intestinal tract was removed. The small intestines (SI) and large intestines (LI) were separated and processed into natural sausage casings as described by Ockerman and Hansen (2000). The SI and LI casings were each cut lengthwise into two sections and each section was cut in half to obtain four portions of each casing.

Before treatment and storage of the casings, about 1 g of each portion was removed for virological analysis as described in the next section, to confirm CSFV presence in the untreated casings.

Each casing part was assigned to one of four treatment groups, which were treated with phosphate or citrate supplemented salt at 4 °C or room temperature of approximately 20 °C).

The phosphate supplemented salt contained 86.5% NaCl, 10.7%, Na_2HPO_4 and 2.8%, Na_3PO_4 (wt/wt/wt). The citrate supplemented salt contained 89.2% NaCl, 8.9% trisodium citrate dehydrate and 1.9% citric acid monohydrate (wt/wt/wt). Saturated solutions of the phosphate and citrate supplemented salt were made in 10 ml of demineralised water and pH values of 9.5 and 4.5 respectively were electronically measured at room temperature (pH-Meter 766 Calimatic, Knick, Berlin, Germany).

All parts were evenly covered with approximately 100 g phosphate or citrate supplemented salt and stored for 30 days according to the respective treatment group.

After storage for 15 and 30 days a sample of about 1 g was taken from each casing portion for virological analysis, as described in the next section. The pH of the samples was also measured electronically after removal of the dry salt and short rinse in demineralised water.

After 30 days of storage a 10 cm long section of casing from each treatment group was mixed with 100 ml phosphate buffered saline (PBS) to obtain a preparation of a neutral pH.

Each pair of piglets received the casing suspension from one of the prepared casing portions subjected to the same treatment, mixed with their regular feed. On day 0 of the experiment each individual animal received 100 g of the prepared mix prior to their normal feed routine to allow for a possible oral infection. Each piglet remained in isolation with daily monitoring of their temperature during a 4-week period. Piglets which died during this period were examined for pathological changes. The remaining animals were euthanized. Venous blood samples were obtained from each animal in duplicate for virological (EDTA tubes No. 09.208.001, Sarstedt, Numbrecht, Germany) and serological (Serum tubes No. 09.207.001, Sarstedt, Numbrecht, Germany) analysis at the start of the 4-week period before the oral infection and after death during or before death at the end of this period.

CSF diagnosis and antibody detection

The protocols were used in this study were those described in the technical part of the EU manual for CSF diagnosis (Commission Decision 2002/106/EC; EURL 2007). Briefly, each casing sample taken was ground in a mortar with 9 ml of Eagle's Minimum Essential Medium (EMEM) supplemented with 10% Fetal Calf Serum (FCS). The supernatant obtained after centrifugation (Heraeus Labofuge

400R, Heraeus Centrifuge Co. Newport Pagnell, UK) of the homogenate for 15 min at 2500 x *g* was used for CSFV isolation as described below.
Leucocytes were obtained by adding 0.5 ml of a 5% Dextran solution to each 10 ml EDTA blood sample taken from the infected piglets and left at room temperature for one to three hours until the leucocytes are visible separated. The supernatant containing the leucocytes was collected and centrifuged 10 min at 437 x *g*, resuspended in 3-5 ml Phosphate Buffered Saline (PBS), centrifuged again and the final pellet was resuspended in PBS and frozen shortly at – 20 °C to lyse the leukocytes.
CSF virus was isolated over two passages by inoculating each casing sample supernatant or leukocyte preparation onto a confluent culture of Porcine Kidney cells (PK15) in EMEM supplemented with 10 % FCS and detected by using the peroxidase-linked antibody assay (Holm Jensen, 1981). The neutralization peroxidase-linked antibody assay limit of detection < 5 $TCID_{50}$) was used to detect CSF antibodies in serum prepared from the blood samples that were already obtained from all piglets (Terpstra et al, 1984; Hyera et al., 1987).

Statistical analysis
A McNemar's test (Petrie & Watson, 2000) was used to compare paired observations of CSFV presence in each piglet before and after oral infection ($P < 0.05$).

Results and discussion

A study done on the thermal and pH stability of different CSFV strains (Depner et al., 1992), showed a mean half-life of 50 hours at neutral pH and storage at 20 °C and a mean half-life of 260 hours at pH 4 and storage at 4 °C. In addition, a previous study (Depner et al. 1998) showed that CSFV can survive in casings when stored in saturated brine (pH 6.3) for a period of 30 days at 4 °C.
Although salted casings can be stored at 20 °C without loss of quality (Bakker et al., 1999), lower storage temperatures (8-12 °C) are generally used by the casing industry (ENSCA, 2008). As a consequence, only the efficacy of the phosphate or citrate supplemented salt was determined at 4 and 20 °C to allow for any storage temperature over 4 °C.
CSFV antigen was confirmed to be present in each portion of intestine at day 0 (prior to treatment and storage). On day 15 of storage CSFV antigen was again found in all portions (Table 1), but on day 30 of storage CSFV antigen was found only in the portions treated with citrate supplemented salt and stored at 4 °C.
Only piglets that were fed preparations of these portions developed a CSFV infection. All four infected piglets showed clear clinical symptoms of the disease with body temperatures > 40.7 °C and CSFV antigen was found in blood samples from these animals.
CSFV antibodies were not detected in the blood samples taken from all piglets before they were fed casing preparations or in the blood samples taken from those animals that did not develop CSFV infections after feeding of casing preparations.
The efficacies of the different treatments of casings were determined by regarding all animals with confirmed CSFV infection as being infected as a result of the consumption of casing preparations and all other animals as uninfected.
The treatment of casings with citrate supplemented salt and storage at 4 °C can therefore be deemed ineffective ($P < 0.05$) for inactivating CSFV in casings,

whereas all other treatments were effective although no distinction could be made between the efficacies of the various treatments.

Table 1: Presence of CSF virus and pH values in pairs of casings treated with phosphate or citrate supplemented salt after storage for15 and 30 days

Salt supplement	Casing type	Storage temp. (°C)	Day 15		Day 30	
			pH	CSFV	pH	CSFV
Phosphate	Small intestine	4	10.3	Pos.	10	-
Phosphate	Small intestine	20[a]	10.7	Pos.	10.3	-
Phosphate	Large intestine	4	10.2	Pos.	10	-
Phosphate	Large intestine	20	10.4	Pos.	10.3	-
Citrate	Small intestine	4	5.3	Pos.	4.8	+[b]
Citrate	Small intestine	20	5.2	Pos.	5.0	-
Citrate	Large intestine	4	5.0	Pos.	5.0	+[b]
Citrate	Large intestine	20	5.1	Pos.	5.1	-

a) Room temperature, approximately 20 °C
b) All animals which were subsequently fed with these samples developed CSFV infections

For the infection model used in this study, a specific scenario was followed to mimic a plausible practical situation. The fattening pig was experimentally infected with the highly virulent CSF virus strain “Koslov” (Mittelholzer et al., 2000; Wehrle et al., 2007) and killed during the prodromal phase of the infection, allowing it to pass slaughterhouse inspection undetected, while CSFV was confirmed in the positive controls (Depner et al., 1997; Moennig et al., 2003). As a low oral dose of a highly virulent CSFV strain can lead to an infection (Dahle & Liess, 1992), all piglets could have become infected if fed with a casings preparation that contained infective CSFV. Results from a similar infection model described by Helwig and Keast (1966), with CSFV by consumption of infected casings indicated that infection is possible, but that virus survivability depended strongly on the processing and storage conditions used with the casings.
Taking into account the combined effects of temperature, pH and prolonged exposure on the survivability of CSFV, the results from this study are in line with the previous findings by Depner et al. (1992). Although only the highly virulent CSF strain “Koslov” was used in this study, it is likely that these results apply to CSFV in general, as a non-strain-specific inactivation treatment was used.
Given the preference to phosphate supplemented salt when processing casings into sausages (Verkleij & Keizer, 2003; Houben et al., 2005; Nakae et al., 2008), and the possibility to use one precautionary treatment against FMDV (Wijnker et al., 2007) and CSFV infection in casings, a recommendation can be made to use phosphate supplemented salt during 30 days of storage, at temperatures over 4 °C.
In order to determine that a treatment with phosphate supplemented salt has been applied an existing phosphate assay kit has been recently validated for casings (Wijnker et al., 2009). Future studies in casings will be needed to determine whether this treatment is also effective against the causative agents of other contagious viral diseases.

Acknowledgments

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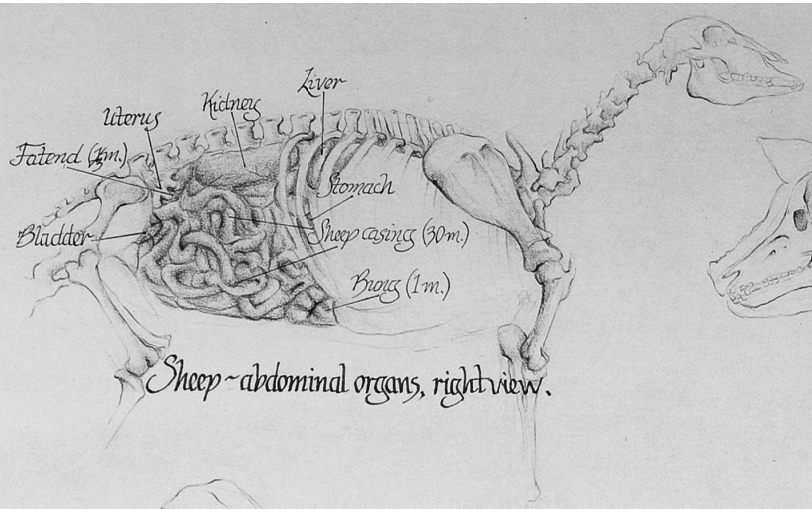
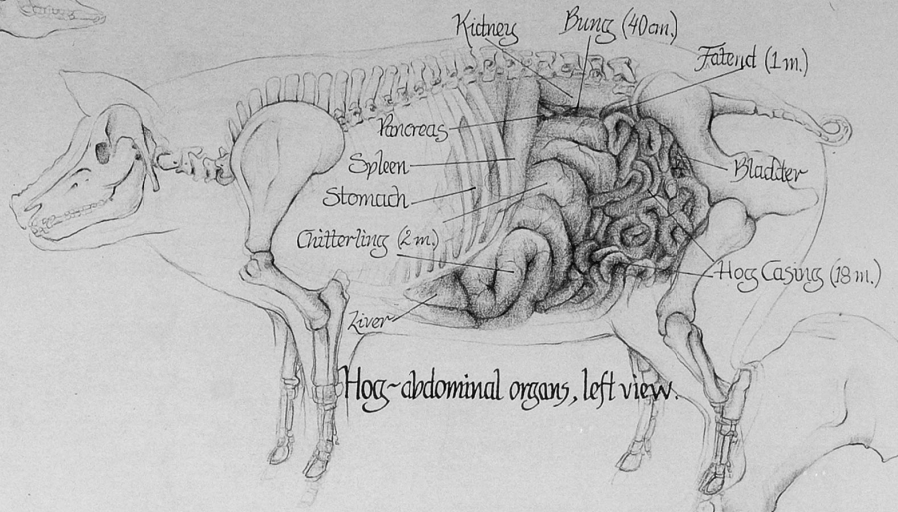
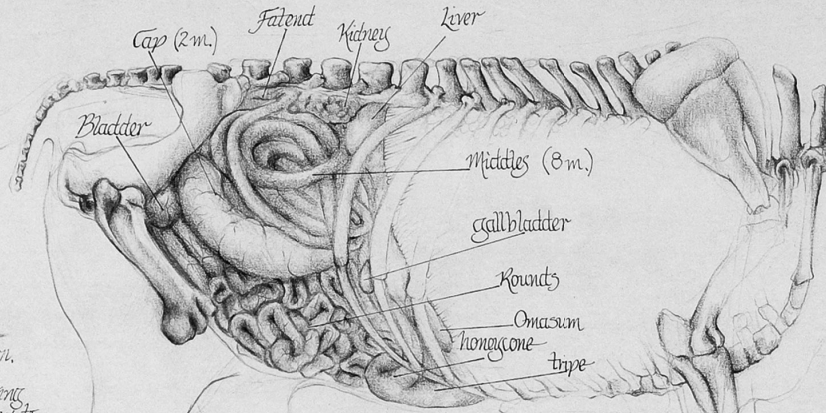


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 7

Phosphate analysis of natural sausage casings preserved in brines with phosphate additives as inactivating agent – Method validation

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Abstract

Certain phosphates have been identified as suitable additives for the improvement of the microbial and mechanical properties of processed natural sausage casings. When mixed with NaCl (sodium chloride) and used under specific treatment and storage conditions, these phosphates are found to prevent the spread of foot-and-mouth disease and classical swine fever via treated casings. The commercially available Quantichrom™ phosphate assay kit has been evaluated whether it can serve as a reliable and low-tech method for routine analysis of casings treated with phosphate. The outcome of this study indicates that this particular assay kit has sufficient sensitivity to qualitatively determine the presence of phosphate in treated casings without interference of naturally occurring phosphate in salt used for brines in which casings are preserved.

Introduction

Previous studies on the usability of certain additives during the processing of natural sausage casings have identified phosphate as a suitable agent. Studies by Bakker et al., (1999) saw a marked improvement on hygienic aspects and mechanical properties of casings treated with phosphate. Especially the effect of trisodium phosphate on the slipperiness of casings during the stuffing process showed a clear improvement (Houben et al., 2005).
Additionally, several studies have been done to determine whether certain additives can play an important role in the prevention of spreading contagious animal diseases via food products, such as foot-and-mouth disease (FMD) and classical swine fever (CSF) (Wooldridge et al., 2006). In 1974 Böhm and Krebs confirmed the efficacy of a 5-min treatment with 0.5% citric acid on infected sheep casings to inactivate the FMD virus. Wijnker et al., (2007) showed that treatments with either salt (NaCl) or phosphate supplemented salt and storage for 30 days at ambient temperature were equally effective in removing a FMD virus infection in hog and sheep casings. This phosphate supplemented salt, in combination with the storage conditions, is also found to be effective against CSF virus in hog casings (Wijnker, et al., 2008).
Both FMDV and CSFV are notifiable to the OIE, the World Organisation for Animal Health (www.OIE.int) and known for their severe economic impact if an outbreak occurs. Generally, diseased animals are destroyed and rigorous actions are taken to prevent the viral spread, although it can be possible that infected animals are slaughtered undetected in a pre-clinical stage of the disease. Secondly the movement of animal products either from the stricken area or surrounding regions is also restricted. Only if an acknowledged treatment can be applied to the respective products, will transport be allowed. For FMDV the treatment of casings with salt has been accepted in Europe (EC Council Directive 2003/85 on community measures for the control of foot-and-mouth disease) and by the OIE. Having a second treatment available using the phosphate supplemented salt as a preventive measure against the spread of both FMDV and CSFV, it is clearly relevant to have a methodology in place that can confirm application of the antiviral treatment. To verify after-the-fact that phosphate supplemented salt was used during the recommended 30-day preservation period, an existing, easy to use phosphate assay kit was evaluated in this study. Based on the results from Houben (2003) it was already known that the initial use of phosphate in treated casings can be detected. However, the elaborate sensitive techniques used by Houben included freeze-drying of samples followed by colorimetric analysis (Rouser et al., 1966) and were therefore not regarded as a suitable method for routine analyses.
The scope of this study was to evaluate the usefulness of the Quantichrom™ phosphate assay kit (DIPI-01K, Gentaur Molecular products, bvba, Brussels, Belgium) on the analysis of phosphate in treated casings. To this purpose the phosphate concentrations and pH values were determined in brines from different origin and a dose-response curve presented on different phosphate concentrations in brine. The final validation was achieved by analysing the phosphate content of casing samples (both sheep and hog), preserved in phosphate supplemented salt with different phosphate concentrations for a minimum of 30 days. The outcome of this evaluation may lead to a recommendation on the applicability of the Quantichrom™ phosphate assay kit as a standard method for the phosphate analysis in treated casings.

Materials and methods

Evaluation of the Quantichrom™ phosphate assay kit

For the detection of phosphate in different brine mixtures or extracts of natural casings, the Quantichrom™ phosphate assay kit was used. In this assay, malachite green dye and molybdate are used to form stable coloured complexes with inorganic phosphate (Ekman and Jager, 1993). The standard assay was performed according to the kit's manual; 100 µl Quantichrom™ reagent was added to 50 µl sample or standard, in a polystyrene flat-bottom 96-well plate. The reagents were gently mixed and incubated at room temperature for 20 minutes. Subsequent colour development was measured on a Bio-Rad benchmark microplate reader (Optical Density (OD) 590, Bio-Rad Laboratories BV, Veenendaal, the Netherlands). Evaluation of the kit was performed by testing standard solutions of 0-50 µM di-sodium hydrogen phosphate (Na_2HPO_4, Merck KGaA, Darmstadt Germany).

Phosphate analysis of different salts used for brines

For this analysis samples of 3 different salts were taken, which are commonly used by the casing industry for the preservation of casings. Sample 1 French origin (F), produced from seawater by solar evaporation techniques; Sample 2, Chinese origin (CN), produced by mining; Sample 3, Dutch origin (NL), produced by mining.

Brines of these salts were made freshly by dissolving 15 or 30 g of the appropriate salt in 100 ml MilliQ water (Millipore, Billerica, MA, USA). The resulting solutions were used undiluted in the Quantichrom™ phosphate assay kit. In addition, two extra samples were prepared of Salt NL (30 g / 100 ml) that was spiked with 30 µM (2.85 mg / l) phosphate.

Phosphate analysis of natural casings preserved in different brine mixtures

Different mixtures of Chinese (CN) and Dutch (NL) salts and phosphates were prepared (Mettler-Toledo PE 360 scale, Tiel, The Netherlands) according to table 1.

Table 1: Different mixtures of NaCl of Chinese and Dutch origin and phosphate salts

Mix	NaCl % [a]	Na_3PO_4 (Mw 164) % [a]	Na_2HPO_4 (Mw142) % [a]
CN-0 / NL-0	100	0	0
CN-0.5 / NL-0.5	93.2	1.4	5.8
CN-1 / NL-1	86.5[b]	2.8[b]	10.7[b]
CN-2 / NL-2	73.0	5.6	21.4

a) Percentages are given as weight (g) percentages
b) Concentrations used by Wijnker et al. (2007, 2008) for the inactivation of FMDV and CSFV

All eight mixtures have a final weight of 100 grams each. The solubility of NaCl is approximately 350 g / litre water (Lide, 2007); therefore from each mixture fully saturated brine was made of approximately 280 ml.

Dry salted natural casings (courtesy Van Hessen bv, The Netherlands) of sheep and hog origin were desalinated in running water and samples of 50 cm in length were prepared. Per casing sample 100 ml brine mixture was used for preservation, creating 16 different test batches and left at room temperature

(RT) during the incubation time of 52 days. The pH of the brine mixtures was measured once a week (Mettler-Toledo 320 pH meter Tiel, The Netherlands). Circular sections of 2 cm in length were taken from the casing samples for analysis. Each section was cut open, divided into 4 equal pieces and residual brine was removed after shaking in 300 ml MilliQ water. Casing pieces were subsequently dried on tissue paper and transferred to a clean Erlenmeyer containing 10 ml MilliQ water. The solution was incubated 15 min at 60 °C in a water bath while shaking. After incubation the casing pieces were centrifuged (120 s, 4000*g*) and the extraction liquid analysed without further dilutions using the Quantichrom™ phosphate assay kit.

Results

Evaluation of the Quantichrom™ phosphate assay kit

A calibration curve was made using stock solutions of Na_2HPO_4 from 0 to 50 µM to check the linearity of the colour development. As is shown in figure 1, the observed response is not completely linear within this concentration range and a second order logarithmic equation was chosen as the correct correlation between optical density and phosphate concentration (depicted in the top right corner of figure 1).
In further experiments a new calibration curve was made for every experiment and the resulting second order equation was used for the calculation of the phosphate concentration of unknown samples.

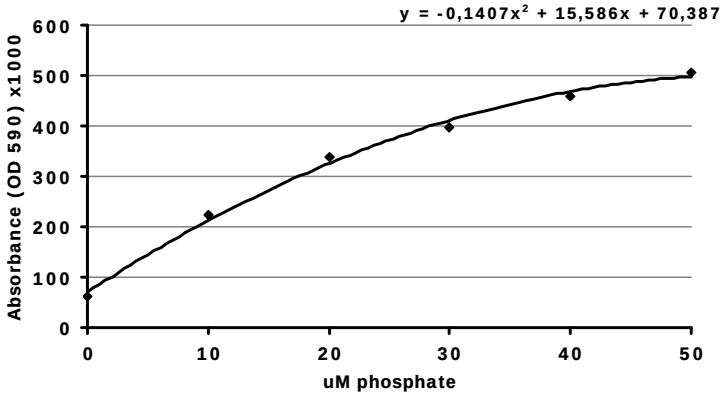

Figure 1: Calibration curve using the Quantichrom™ phosphate assay kit

pH measurements of brines with phosphates added

Single pH measurements were taken weekly of each brine in which the natural casings were incubated over a total period of 52 days (Figure 2). No significant change in pH was recorded at any of the eight measurements for each of the different brines.

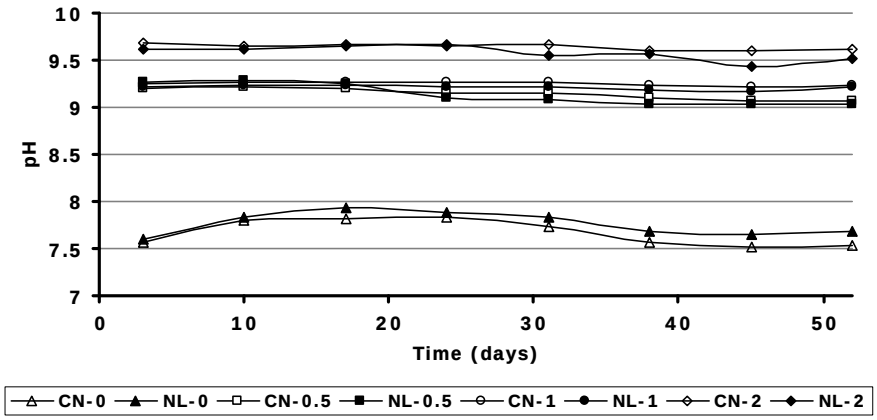

Figure 2: Changes in pH measurements over a 52-day period in different brines according to table 1

As expected, the pH values of brines containing phosphates at different concentrations (average 9.2 / 9.3 / 9.6) are higher than brines made from sodium chloride alone (average 7.7).

Phosphate content of different salts used in brines

In total six different brines were made from the described salt samples (F, CN, NL), in order to determine the naturally occurring phosphate content of these salts. From each brine 5 separate samples were subsequently analysed (Table 2).

Table 2: Phosphate content of brines made from French (F), Chinese (CN) and Dutch (NL) salt samples

Brine origin & conc. gram / 100 ml	µg phosphate / gram salt
F 15	4.2 ± 0.4
F 30	3.8 ± 0.2
CN 15	-0.3 ± 0.6
CN 30	-0.4 ± 0.1
NL 15	-0.6 ± 0.1
NL 30	-0.2 ± 0.2
NL 30 spiked[a]	6.4 ± 0.2

The results show that only Salt F (solar evaporation) contains a detectable amount of naturally occurring phosphate. All other samples had non-detectable phosphate concentrations.

a) Salt NL 30 is spiked with 30 µM phosphate (corresponding to 9.5 µg / gram salt)

The phosphate concentration of 3.8 ± 0.2 µg phosphate (PO_4) per gram salt for the 30 g / 100 ml solution and the comparable 4.2 ± 0.4 µg / g for the 15 g / 100 ml solution corresponds to 0.0004% weight content of phosphate in the French salt.
The amount of phosphate measured in the spiked sample (*n* = 2) was 6.4 ± 0.2 µg phosphate per gram salt. This is approximately 30% lower than the actual amount of 9.5 µg phosphate per gram salt added to the sample.

Phosphate content of brines with different concentrations of phosphates added

The phosphate content (*n* = 3) of each Chinese (CN) or Dutch (NL) brine was tested as an extra control for both the Quantichrom™ phosphate assay kit and the general experimental setup.

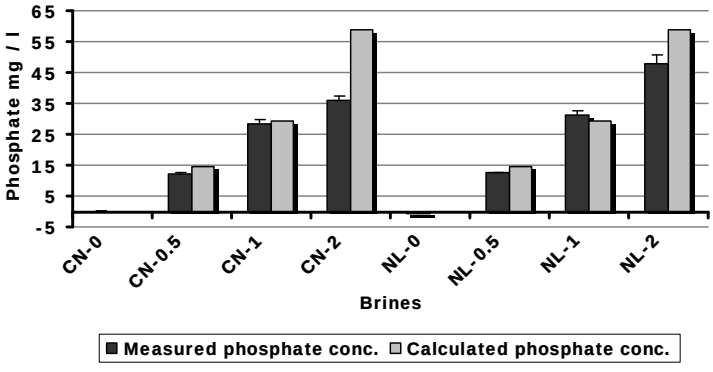

Figure 3: Measured and calculated phosphate concentrations in different brines according to table 1

Figure 3 shows that the measured phosphate concentration of the brines corresponds well with the calculated concentrations, except for the brines containing the highest phosphate concentrations where a lower phosphate content is measured.

Phosphate content of hog and sheep casings preserved in brines with different concentrations of phosphates added

Hog and sheep casings (*n* = 3) were preserved in brines containing different amounts of phosphate for 52 days. Subsequently, the presence of phosphate in the casings was determined using the Quantichrom™ phosphate assay kit. Figure 4 shows that storage of casings in brines containing phosphate clearly results in a detectable amount of phosphate in the casings themselves.

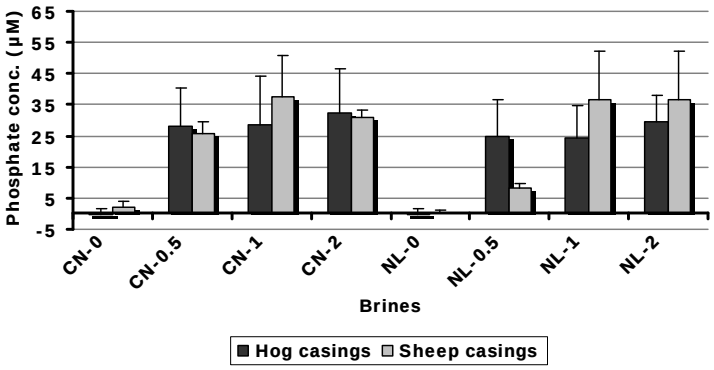

Figure 4: Phosphate content of hog and sheep casing extracts stored in different brines according to table 1

For the highest two phosphate concentrations tested in both hog and sheep casings, phosphate levels high in the detection range of the Quantichrom™ phosphate kit were extracted from the casing. However, no clear correlation between initial phosphate concentration of the respective brines (Figure 3) and measured extracted phosphate from the casings could be determined.

Discussion

The scope of this study was to determine whether the Quantichrom™ phosphate assay kit would be more appropriate as a routine method for the analysis of phosphates in treated casings compared to the method described by Houben (2003). Secondly, the kit was evaluated on whether it could distinguish naturally occurring phosphate concentrations in salt from added phosphates used for the treatment of casings. According to the results, the Quantichrom™ phosphate assay kit is a very sensitive method to detect phosphate in liquid samples. Samples containing 10 µM phosphate can be detected reliably and are within the detection range specified by the manufacturer of the kit. However, the linear correlation between OD and phosphate concentration described in the manual between 0 and 50 µM could not be reproduced in this study. Instead a second order logarithmic function better fit the calibration data and therefore this equation was used for quantification of unknown samples. As a new calibration curve was made for every experiment and the resulting second order equation was used for the calculation of the phosphate concentration of unknown samples, the outcome of the final results can be regarded as reliable.

Only one of the tested salts (Salt F) used for brines contained a detectable amount of phosphate (0.0004% wt/wt). However, the low amount of naturally occurring phosphate in this salt is negligible when compared to the concentrations of phosphates intended for the inactivation of FMDV and CSFV (2.8% wt/wt Na_3PO_4 and 10.7% wt/wt Na_2HPO_4).

From the results depicted in figure 2, it can be concluded that the pH of the brines did not significantly change after 52 days of storage at room temperature. A non-quantitative correlation between phosphate content and final pH is observed showing that (as expected) addition of higher amounts of Na_2HPO_4 and

Na_3PO_4 to NaCl leads to higher pH (>9.0) values of the brine. The pH values found in this study correspond to those found previously in the FMDV (pH 10.0) and CSFV (pH 9.5) inactivation studies (Wijnker et al., 2007, 2008). This further confirms that the testing conditions used in this method validation study were comparable to the inactivation studies which will be in support of the final conclusions.
Although the assay kit is deemed accurate and sensitive for phosphate concentrations up to 47 µM, Chinese (CN) and Dutch (NL) salt samples showed a negative phosphate concentration at low concentrations. This could reflect the error of the calibration curve at very low phosphate concentrations, although correct standards were included in the experimental setup. A second possibility is that high salt concentrations could interfere with the colour development giving an underestimation of the true phosphate content. To detect the magnitude of the possible error, the 30 g / 100 ml NL brine was spiked with 30 µM Na_2HPO_4 and subsequently analyzed. The resulting value of 20.2 ± 0.6 µM shows that an underestimation of 30% could occur in high salt solutions, although it does show that even in these high salt solutions minute traces of phosphate are detected. Therefore, all samples with a negative value for phosphate content can be considered zero.
The results of figure 4 suggest a significant difference in phosphate content of the respective casing extracts, although the high standard deviations for all phosphate / NaCl mixtures prevent an accurate distinction between the different phosphate concentrations used. However, the phosphate / NaCl mix (CN/NL-1) used for FMDV and CSFV inactivation is clearly distinguishable from the situation were no phosphate salt mixture was used (CN/NL-0).
Standard preservation and storage of casings is either in dry-salt or fully saturated brine. In both the FMDV and CSFV inactivation studies, casings were stored in dry-salt, using the described phosphate / NaCl mix. This study used a phosphate / NaCl brine for preservation and prolonged storage of casings (> 30 days) at ambient temperature was maintained. The methodology used in this study for the preparation of casing samples allow both dry-salted or casings stored in brine to be analysed, as it relies on the phosphate content of the treated casings and not its preservation or storage medium. In practice, this will allow for testing of all natural casings subject to confirmation of the required treatment.

Conclusions

Overall, it can be concluded that the Quantichrom™ phosphate assay kit is a useful and reliable tool with sufficient sensitivity to detect naturally occurring phosphate in salt and to qualitatively determine the phosphate concentrations used in treated casings. With a minimum of equipment (microplate reader, water bath and normal glassware) the phosphate content of casings can be determined within 3-4 hours. Therefore this assay kit can be recommended as a new standard method for phosphate analysis in treated casings.

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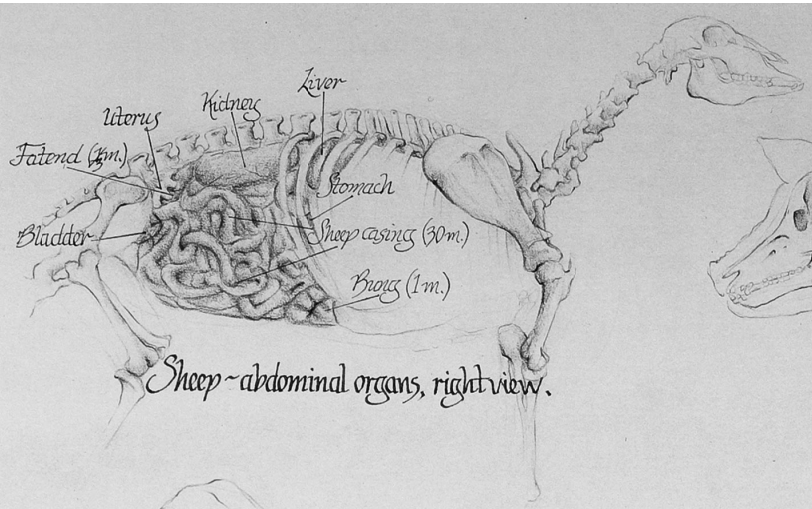
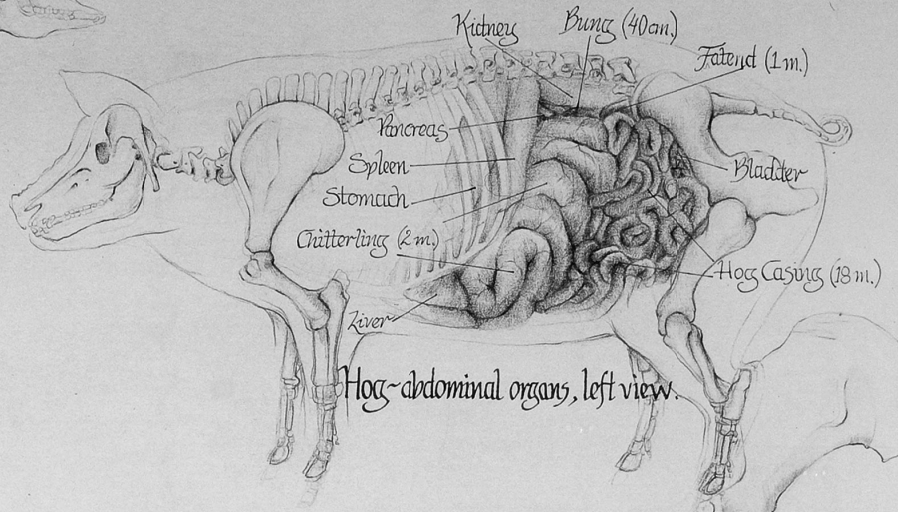
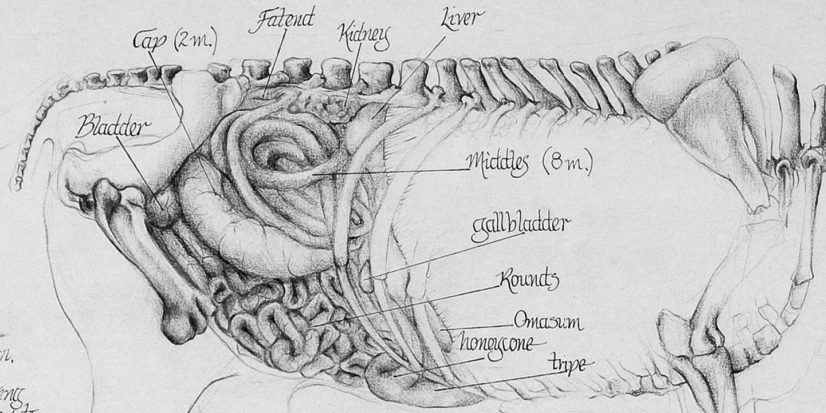


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Profush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40°F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

CHAPTER 8

Summarising discussion

Natural sausage casings are produced from the intestines of various species and used as edible containers for many different types of sausage around the world. As such, casings are regarded fit for human consumption and therefore must meet all food safety and hygiene requirements that apply to food of animal origin. Casings are intrinsically linked to sausages, although casings only constitute 1-1.5% of the actual sausage weight. From this perspective it becomes clear that casings have received very little scientific attention due to the simple fact that a hazard analysis of sausages would either focus on the final product or on the meat batter stuffed inside the sausage. As a result many studies are available on the (anti-) microbiological developments in sausages and only a handful on casings.

Food safety requirements

Since January 1st 2006 the new European hygiene rules became applicable, with Regulation (EC) No 852/2004 (European Commission, 2004a), on the hygiene of foodstuffs and Regulation (EC) No 853/2004 (European Commission, 2004b), laying down specific rules for food of animal origin, as key elements.
Maintaining sufficient hygiene control during the production of foodstuffs is now based on the Hazard Analysis Critical Control Point (HACCP) principles: each step during the processing of casings is evaluated according to this principle and adequate measures taken to assure the safety of the product are mandatory. Additionally, cleaned, scraped and salted casings were given the status of treated products under Regulation (EC) No 853/2004. This provided a clear distinction from fresh meat and meat products that require even more stringent hygiene and production rules. However, this distinction also provides a challenge as casings were now regarded as a separate entity and all hazard evaluations had to be based on data derived from casings.
The subjects studied in this thesis serve therefore a clear purpose: to understand in greater detail the hazards that are included in the production of casings. Whether these hazards can develop into potential food safety risks depends on whether identified quality parameters are maintained at sufficient level during the processing of casings.
Microbiological contamination, resulting in product spoilage or possible outgrowth of pathogenic bacteria is only one of the issues that have been addressed. Implemented European legislation on the prevention, control and eradication of certain Transmissible Spongiform Encephalopathies or TSEs (European Commission, 2001) has set strict requirements on the absence of certain organs and tissues in food of animal origin. Therefore, the potential presence of TSE prions and related tissues in the intestinal tract of ruminants required specific attention.
A second dimension to the thesis was added due to the nature of the product and its global use in sausage production. Derived from the intestinal tract of animals slaughtered around the world, casings are processed at various geographical locations and subsequently shipped to sausage producers in yet other countries. This trans-global movement of casings meant that restrictive measures, implemented to prevent the spread of contagious animal diseases, also apply to casings. The World Organisation for Animal Health (OIE) monitors all notifiable animal diseases and has issued clear recommendations to control the animal disease status in regions or countries of its members. Based on the OIE's impact

assessments the possible inactivation of foot-and-mouth disease (FMD) and classical swine fever (CSF) in casings was studied in more detail.

Preservation of casings

Natural sausage casings are predominantly produced from the intestinal tract of ruminants and pigs, processed in batches and can therefore be extensively contaminated with pathogenic and non-pathogenic bacteria (Riha and Solberg, 1970; Gabis and Silliker, 1974; Wirth, 1990, 1994; Koolmees and Houben, 1997; Bockemühl, 2000; Houben, 2005). Casings are regarded fit for human consumption and must therefore meet the legal and hygienic requirements set for consumable products of animal origin.
In the production of casings salt has been in use for centuries as the main preservative agent and to determine whether this technique can meet the current criteria on food safety, the study described in **Chapter 2** was done. Several brines with increasing salt concentrations and dry salt were tested on their efficacy to eliminate predetermined bacterial loads in casings. As salt concentrations are directly linked to water activity levels (a_w-levels) clear parameters were set to determine the death rates of these bacteria.
Overall it can be stated that Gram-positive bacteria are less sensitive to lowered a_w than Gram-negative bacteria. This is consistent with the fact that Gram-positive bacteria are better equipped to cope with osmotic stress than Gram-negatives (Mellefont et al., 2003). Gram-positive bacteria have constitutive transport systems for the uptake of compatible solutes, whereas Gram-negatives need to implement transport systems (Mellefont et al., 2003). Until this implementation has taken place, accumulation of potassium and its counterion glutamate is used to maintain the turgor (Sleator et al., 2003; Mellefont et al., 2003). The accumulation results in high intracellular levels of potassium glutamate, possibly impairing enzyme function. Furthermore, Gram-negative bacteria display a relatively low turgor, giving a more severe plasmolysis during osmotic stress (Gutierrez et al., 1995). This impairment of enzymes together with the more severe plasmolysis gives an explanation for the higher death rates observed for Gram-negative bacteria at lower a_w.
Previous findings by Gabis and Silliker (1974) on the survival of *Salmonella spp*. in salted casings showed a decrease of approximately one log-cycle in three days in saturated brine (0.75 a_w). A death rate of 0.33 day^{-1} can be calculated, which corresponds to our findings.
A study on the survival of *Listeria monocytogenes* (Nolan et al., 1992), recorded a reduction of five log-cycles in seven days at 0.91 a_w in TSB-YE and added sodium chloride. This is a markedly higher death rate than was observed in our study, but the data from the Nolan study were obtained at a_w levels in the bacterial growth range whereas lower a_w ranges as reported by Uzelac and Stille (1977), will reduce the death rate. These findings correspond to our findings where *L. monocytogenes* survived for up to 30 days at an a_w-level of 0.85.
Death rates of *Staphylococcus aureus* were constant for all investigated a_w-levels. *S. aureus* is known to be the most salt-tolerant non-halophilic eubacterium, and can grow at a_w-values as low as 0.86 (Abee and Wouters, 1999; Gutierrez et al., 1995). This is due to the highly effective transport systems of *S. aureus* for compatible solutes. Nevertheless, a severe decrease in viable cells was observed over the 30-day period. The reason for this subsequent cell-death could be found in a shortage of compatible solutes or substrate for

compatible solutes, since *S. aureus* needs exogenous supply of these substances (Gutierrez et al., 1995).
Shortage of energy can be another factor leading to death of the bacteria, since many mechanisms required to maintain turgor are energy-dependent (Verheul et al., 1997; Abee and Wouters, 1999).
No clear reduction in the survival of *Clostridium perfringens* in the relation to any a_w-level was observed. After inoculation the vast majority of cells died, probably due to the presence of oxygen, since *C. perfringens* is an obligatory anaerobe and very sensitive to the presence of oxygen (Trinh et al., 2000). This left only a few cells to sporulate, which were re-grown using the enrichment procedures described in Chapter 2. Due to the fact that *C. perfringens* is a natural inhabitant of the intestinal tract of many animals (Brynestad 2002), the *Clostridium* spores were not only found during the casing processing (Ockerman and Hansen, 2000), but their presence was also confirmed in salted natural casings, sometimes at high concentrations (Houben, 2005). The recurring re-growth of bacterial spores as found in our study clearly demonstrates the resistance to low a_w-levels of *Clostridium* spores.
The temperature at which the casings were stored (20±1.5 °C) was within the range that provides the highest tolerance against osmotic stress (Mellefont et al., 2003). The measured pH was close to neutral during the storage of the casings and no detrimental influence was to be expected. The results found in this study may therefore represent the maximal survival of specific pathogens in salt-preserved casings.
In general the casing industry uses either dry salt (a_w-level 0.75) or saturated brine (a_w-level between 0.75 and 0.80) for preservation and the storage period will exceed the minimum required 30-day period to eliminate all vegetative bacteria that can be present in casings. By recommending a minimum storage period of 30 days at ambient temperatures and using the water activity level to monitor the saturation level of brine, clear, measurable, parameters are set to maintain an adequate preservation of casings, well below any implemented microbiological critical limits.

Clostridia spp. play a remarkable role in the preservation of casings. Not only that they are ubiquitously present, but also due to the fact that they survive the standard preservation technique of casings using salt or brine, due to their spore-forming capabilities. A large variety of *Clostridia spp*. are present in the intestinal flora of ruminants and pigs (Fink-Gremmels, 2008) mostly non-pathogenic although *C. perfringens* and *C. botulinum* can be present and are regarded as the most commonly involved species in food-borne illnesses (EFSA, 2005). In animal husbandry the usage of antibiotics are more and more restricted to prevent unwanted residues in products of animal origin and to reduce the development of antibiotic resistance in bacteria, but also to prevent the usage of antibiotics as animal growth stimulant. As a result, intestinal flora can increasingly contain *Clostridia*, which will end up in the processed and salted casings as spores (> 1000 cfu / g, Houben, 2005). An increase of spore counts in casings is already being observed throughout the casing industry and the global sourcing and batch-wise production of casings make it virtually impossible to prevent cross-contamination and subsequent spread throughout the production chain. This compelling situation has prompted the study described in **Chapter 3** in order to find new means for the preservation of casings.
A likely substance to achieve this effect is nisin, a bacteriocin produced by certain strains of *Lactococcus lactis subsp. lactis* (Mattick and Hirsh, 1947; Schleifer et

al., 1985), with a known broad-spectrum inhibitory effect on gram-positive bacteria, including spore-forming bacteria and their spores (Hurst, 1981; Davies et al., 1999).
The effect of nisin against spores is more likely to be sporostatic than sporocidal (Delves-Broughton et al., 1996), and has been suggested to be the result of binding to sulfhydryl groups on protein residues (Ando, 1979, Morris et al., 1984). Meghrous et al. (1999) recorded a 10-day delay in *Clostridium* spore outgrowth after exposure to nisin, whereas sporocidal effects of nisin have been observed by De Vuyst and Vandamme (1994) after spores were damaged by heat treatment (3 minutes at 121 °C).
In this study gamma irradiation was applied to destroy all bacteria and spores present on the casings used for the respective models, as studies by Trigo and Fraqueza (1998) and Chawla et al. (2006) had already identified this radiation dose to be sufficient for the destruction of all *Clostridium* spores and vegetative bacteria in casings. The usability of gamma irradiation at 10 kGy depended not only on the efficacy to destroy microbial contamination to create a “clean” model but also whether this treatment would damage the casing beyond practical use. Different casing samples taken after gamma irradiation were tested on various quality parameters, which revealed no detrimental effect of the treatment. As a result it was concluded that gamma irradiation at 10 kGy can effectively inactivate any bacteria or spores present on the casings without quality loss.
Results showed that nisin can reduce the outgrowth of *Clostridium* spores, inoculated at concentrations of 5.10^3 and 5.10^4 spores per gram casing, over a period of 30 days by approximately 1 $\log_{10}$. This reduction was achieved within 8 days after inoculation (second time point for analysis) with similar results for nisin concentrations of 50 and 100 µg / ml water at this and the remaining time points.
This 30-day delay in spore outgrowth was substantially longer than the 10-day delay previously recorded by Meghrous et al. (1999) for several *Clostridia* strains, but it remains to be determined what the full inhibitory period will be.
The results from the binding and availability experiments which were also included in this study, indicated that nisin is bound to the casing wall, whilst exhibiting bactericidal activity away from the casing wall. Previous studies have clarified that nisin binds to glutathione (GSH) in a non-enzymatic reaction (Stergiou et al., 2006), with the free sulfhydryl group of GSH as the proposed binding site for nisin (Rose et al., 1999). In addition it has been shown that the inhibiting mechanism for spore outgrowth is also due to nisin binding to sulfhydryl groups in the envelopes of germinating spores (Morris et al., 1984; Liu and Hansen, 1990). It is therefore quite likely that the sulfhydryl groups in the casing wall and on the spore surface are in competition to bind the available nisin.
As mentioned before, the results from the availability study show bactericidal activity away from the casing wall, by producing inhibitory zones around uniform casing disks containing nisin and placed on agar plates inoculated with *C. sporogenes*. However, it is unclear whether this was achieved by nisin that was bound reversibly to the casing wall and subsequently released or by unbound nisin that diffused unhampered away from the casing disk as all sulfhydryl groups were already occupied. Assuming the latter, a nisin concentration is required above the Minimal Inhibitory Concentration for *C. sporogenes* (38.4 µg / ml, Meghrous et al., 1999), combined with the amount needed to saturate the sulfhydryl groups in the casing disk. Absence of any inhibitory zones surrounding the paper disks at nisin concentrations lower than 50 µg / ml and only an

inhibitory zone surrounding the casing disks at the highest nisin concentration (100 µg / ml) are in line with this assumption.
Based on the findings described it can be said that nisin can augment the preservation of casings to reduce the outgrowth of spores present on casings. However, any recommendation for a practical implementation of nisin in the preservation of casings can not be made. The binding of nisin to the casing wall and the achieved reduction of approximately 1 $\log_{10}$ cfu / g casing are two factors that exclude a recommendation on usability. This said, empirical data from the casing industry suggest that an application of nisin during the processing of casings does reduce spore outgrowth, although it remains to be determined whether this will be a viable option.

As the casing industry is a traditional industry, it relies on highly skilled manual labour for most of the production process and uses the ancient technique of salting as its principal means of preservation. To add to the awareness on food preservation, known parameters should be used. In this respect the concept of Hurdle Technology (Leistner, 2000) is readily applicable to the casing industry.
This concept applies various gentle preservative methods (called hurdles) into an effective combination to maintain microbial safety and stability as well as sensory and nutritional quality of food stuffs.
To measure the salt concentration in brine, a salinometer with Baumé scale is commonly used to measure the specific gravity of the salt solution. Although a practical instrument, the Baumé scale has very little reference to available scientific literature. By introducing water activity as a replacement, an internationally accepted standard is used with ample references and inhibitory values for relevant micro-organisms. Together with temperature and pH, water activity is one of the key hurdles used in food preservation.
Table 1 (Fink-Gremmels, 2008) is included to illustrate the practical usability of water activity as quality parameter to assess the potential risk of different micro-organisms in salted casings. These data indicate that most bacterial species, including the ones that can be found in the gastrointestinal tract of ruminants and pigs, cannot survive a water activity level below 0.91.

a_w	**Micro-organisms generally inhibited by a_w at this point**
0.950	*Pseudomonas, Escherichia (E. coli), Proteus, Shigella, Klebsiella, Bacillus, Clostridium perfringens*, some yeasts
0.910	*Salmonella, Vibrio parahaemolyticus, C. Botulinum, Serratia, Lactobacillus, Pediococcus*, some moulds, *Rhodotorula, Pichia*
0.870	Many yeasts (*Candida, Torulopsis, Hansenula*), *Micrococcus*
0.800	Most moulds (mycotoxigenic penicillia),*Listeria monocytogenes, Staphylococcus aureus*, most *Saccharomyces (bailii) spp., Debaryomyces*
0.750	Most halophilic bacteria, mycotoxigenic aspergilli
0.650	Xerophilic moulds (*Aspergillus chevalieri, A. Candidus, Wallemia sebi*), *Saccharomyces bisporus*
0.600	Osmophilic yeasts (*Saccharomyces rouxii*), few moulds (*Aspergillus echinulatus, Monascus bisporus*)
0.500	No microbial proliferation

Table 1: micro-organisms inhibited at various a_w values

The usage of salt as main preservative agent for casings can also pose a problem. Sausage producers need to prepare well in advance the quantities of casings for their stuffing processes. Extensive soaking to desalinate and rehydrate the salted casings is required in order to meet their quality demands for the production of sausages. The presence of high salt concentrations in brine can also interfere with other preservative agents, by changing solubility or by complex-forming rendering the added agent inactive. Insufficient information is currently available to answer these questions, either in laboratory experiments or industrial trials.
Additionally the presence of high salt concentrations becomes more and more an issue in waste water management and steps will be required to reduce it. Last but not least, the international transport of casings in dry-salt or brine means that a substantial volume and shipped weight are being used by the added salt and water. Different preservation or storage techniques could improve this situation, resulting in lower fuel costs and lesser greenhouse gas emissions during transport. However, it will be up to the international casing industry to determine if and when these issues become relevant and what the common strategy will be, as major changes in the preservation of casings will have a global impact, involving all steps in the production process of casings.

Bovine Spongiform Encephalopathy

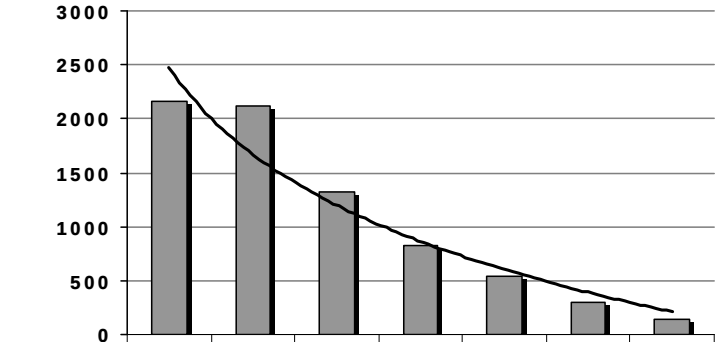
In the production of beef casings from small intestines, known as “beef rounds”, only the duodenum and jejunum are included as the ileum has an aberrant shape and texture and is enclosed in mesenteric fat. From a technical view-point it is therefore unfit for casing production and is removed as standard operating procedure. After the cleaning process all intestinal tissue layers (serosa, musculary layer, submucosa and mucosa) can still be identified in beef casings, unlike sheep or hog casings that consist only of submucosa.
Embedded in these tissue layers, the neural and lymphoid tissue can be regarded as marker tissues for the potential presence of Bovine Spongiform Encephalopathy (BSE) disease-specific prion protein (PrP^{Sc}). **Chapter 4** describes a quantitative histological analysis of bovine small intestines before and after processing into casings. Results showed that a weight reduction of about 50% is achieved during standard cleaning procedures, whereas 90% of the mucosa and 48% of the lymphoid tissue and no neural tissue were removed. Based on the quantitative histological image analysis, it was calculated that 1 m of cleaned casings, weighing on average 64 g, contains about 2.8 g of mucosa, 0.3 g of lymphoid tissue and 0.1 g of neural tissue. From a consumers’ perspective, these data indicate that approximately 0.09 g lymphoid and 0.02 g neural tissue would be consumed per 200 g sausage produced in beef casings.
The presence of lymphoid tissue (lymphonoduli aggregati or Peyer’s Patches and lymphonoduli solitarii) and neural tissue (plexi of Meissner and Auerbach) in cleaned beef casings does not implicate an inherent risk in transferring BSE, as PrP^{Sc} need to be present in these tissues.
Due to a different cleaning process sheep casings are considered to have a negligible BSE infectivity risk as Peyer’s patches are removed completely. From the neural tissue, only the plexus of Meissner will remain, as it is part of the submucosa layer that solely constitutes sheep casings (Koolmees et al., 2004). In sheep, contrary to cattle, only the ileum is designated specified risk material

(SRM) and removed as a precautionary measure (European Commission, 2001) although BSE remains unconfirmed in sheep (EFSA, 2007, 2008a).
Contrary to the pathogenesis in sheep and mice, BSE infectivity in cattle has, up to now, an exclusive intraneuronal spread from the intestinal tract to the central nervous system (Buschmann and Groschup, 2005; Hoffmann et al., 2007). Only in some of the clinically affected, experimentally induced and naturally occurring cases of BSE could sparse immunostaining of neurons in the myenteric plexi and disease-specific prion protein (PrP^{Sc}) be detected in the Peyer's patches of the distal ileum (Wells et al., 1994; Terry et al., 2003). In cattle incubating BSE, PrP^{Sc} was only detected in the Peyer's patches of the distal ileum, not in the myenteric plexi of the distal ileum or elsewhere (Hoffmann et al., 2007).
Using a similar argumentation based on available literature, the U.S. Food Safety and Inspection Service (FSIS) amended in October 2005 its rule ''Prohibition of the Use of Specified Risk Materials for Human Food and Requirements for the Disposition of Non-Ambulatory Cattle'' (US Food Safety and Inspection Service, 2005). This amendment re-allowed the use of beef casings for the production of sausages originating from countries with a GBR II categorisation, indicating a controlled BSE risk according to World Organisation for Animal Health (OIE) standards (World Organisation for Animal Health, 2008). A critical point in the FSIS amendment and OIE's Terrestrial Animal Health Code is the complete removal of the distal ileum from bovine intestines and prohibited use in the preparation of foodstuffs. As mentioned above, this requirement is already fulfilled, as the ileum is unfit for casing production and removed as standard operating procedure (ENSCA, 2006).
The results from this study clearly illustrate the limited presence of lymphoid tissue in cleaned beef casings, by incidence and calculated amount. As BSE infectivity remains unconfirmed in both lymphoid and neural tissue present in the intestinal tract used as beef casings and the final exposure to the consumer is extremely limited, it can be suggested that beef casings carry a negligible risk in transferring BSE. Additional research using beef casings originating from BSE-infected cattle could further corroborate these findings.

BSE was first officially diagnosed in the United Kingdom in November 1986 and became quickly known as "mad cow disease" due to the typical movement and behaviour of animals suffering from the disease.
Since the first BSE outbreak in 1986, more than 180.000 cases in cattle have been reported in the UK alone. Epidemiological models predicted a rapid dissemination of the disease between animals via contaminated meat and bone meal in feed, and a considerable increase in number of affected consumers was envisaged due to an established link between BSE and new variant Creutzfeldt - Jakob disease (vCJD) in humans (1996).
As a consequence, drastic measures were taken by the European Union authorities to ensure consumers' safety, including the designation of beef casings produced from European bovine intestines as SRM and therefore no longer fit for human consumption (European Commission Scientific Steering Committee, 1997, 2000). This precautionary ban terminated the production of beef casings in Europe and allowed only imports into the European Union from countries with a negligible BSE risk (European Commission, 2001).
In contrast to these first epidemiological predictions, the number of reports of BSE in the UK began to decline in 1992 and continuously declined year by year since then, not only in the UK but in the entire EU. Additionally the number of affected consumers did not reach the predicted dramatic levels. Although a

fortunate development, sceptics still debate whether this was a result of the implemented restrictions or an overestimated doom scenario to begin with.

Figure 1 (EFSA, 2008b) gives an overview of the number of BSE cases detected through the BSE surveillance and the culling of animals in the framework of BSE eradication measures in the EU member states (EU 15, prior to 2004) during the period 2001-2007.



	2001	2002	2003	2004	2005	2006	2007
BSE cases	2164	2118	1325	818	536	305	145

Figure 1: Number of detected BSE cases in EU 15 during the period 2001-2007

As a result of this steady decline of BSE cases in Europe, the option became available to review the potential BSE risk of products currently included in the SRM list.

In the EU member states production and import conditions of beef casings are ruled by the BSE / TSE regulation (European Commission, 2001) stating that certain meat products can only be produced or imported into the EU if the country of origin has a BSE negligible status. Cattle intestines, from duodenum to rectum, are listed as SRM and most European member states have a BSE controlled status, disallowing the production of beef casings in the EU.

Using the quantitative histological results for beef casings described in chapter IV, a BSE infectivity risk - consumer exposure assessment can now be made. Preliminary results from such an assessment indicate that the potential risk when consuming a sausage produced in beef casings is far less than consuming a T-bone steak from the same animal (ENSCA, 2008).

In different scientific publications, the presence of BSE prions has only been confirmed in the distal ileum as part of the intestinal tract of cattle. However, various criticisms have been given on the small number of animals involved in these studies, resulting in insufficient data for statistical analysis. To address this issue, a possible scenario would be to obtain the small intestines of experimentally and naturally infected cattle, have it processed into beef casings according to the industry's standard operating procedures and subsequently analysed for the presence of BSE prions. However, the feasibility of such a study is yet unknown, given the inherent difficulties on the availability of infected intestines and subsequent processing and analysis.

Infectious animal diseases in casings

The international trade of casings as a foodstuff is ruled by strict animal health and hygiene requirements, included on the available veterinary health certificates used around the world. As most countries are members of The World Organisation for Animal Health (OIE), specific trade restrictions are often based on the OIE's recommendations if and when an outbreak of a contagious animal disease occurs. In addition to the OIE's Terrestrial Animal Health Code, different countries and regions have implemented their own measures and requirements

to deal with possible outbreaks. The enormity of the economical impact due to such outbreaks can be illustrated by two recent European examples: Foot-and-mouth (FMD) outbreak United Kingdom 2001, total costs £ 8 billion (Anderson, 2007); Classical swine fever (CSF) outbreak the Netherlands 1997, total costs € 1.4 billion (Ministry of Agriculture, the Netherlands, 2007).
As a result from the absence of specific data for FMD and CSF virus survival in processed casings, risk assessments for the international trade in casings have been extrapolated from other products, with either insufficient risk reduction or resulting in unnecessary trade restrictions. However, to determine the efficacy of certain methods available for the inactivation of micro-organisms, only the product itself can serve as its own model to make an objective assessment (Park and Beuchat, 2003).
Chapters 5 and 6 describe the studies done to determine the efficacy of FMD and CSF virus inactivation in cleaned casings, either by salt (NaCl) or by using phosphate supplemented salt. In the review article by Alexandersen et al. (2003) on the pathogenesis and diagnosis of FMD, a clear overview is presented of the primary sites of infection and secondary sites of replication. Based on the information presented it can be suggested that the FMD virus will primarily replicate in tissues of embryonic ectodermal origin (Noden and De Lahunta, 1985), mainly oral cavity and skin. Based on the endodermal origin of the intestinal tract it can therefore be suggested that the virus titre found in cleaned casings is most likely based on the minimal amount of blood remaining after processing. This is supported by Oleksiewicz et al. (2001) on the quantification of FMD virus (FMDV) in various porcine tissues. In this study the following observations were made: Tissue titres of heart (mesoderm), kidney (endoderm), liver (endoderm) and spleen (mesoderm) were remarkably uniform (10^{2}-10^{3} $TCID_{50}$) and distinctly lower than the peak viraemia titre of 10^{6}-10^{7} $TCID_{50}$, found 2 days post-infection. Both liver and spleen are likely to be involved in the active clearance of viraemia by phagocytosis. Therefore the FMDV concentrations found in these tissues probably represent the absolute maximum which could be attained by serum contribution and sequestration of virus. As a result, higher virus concentrations are more indicative of active FMDV replication and were found in tissues of ectodermal origin. This may account for the consequently low FMDV titres found in cleaned casings, not only in our animal experiments (max. $10^{3.0}$ pfu / ml) but also in the original study by Böhm and Krebs (1974).
Additionally, Alexandersen et al. (2003) reported on the survivability of FMDV. Of great interest is the biphasic kinetic curve for the decay of FMDV infectivity. As shown in our results of the cell culture virus titre reduction at pH 10, an initial steep decay is followed by a prolonged, shallow tail of residual infectivity.
According to Bakker et al. (1999), storage at temperatures below 20 °C does not significantly improve the quality of the salted casings and is therefore not included in the standard procedures of the industry. Results from our study showed no positive samples after 30 days for the salted casing samples. This indicates that the time set for minimal storage is sufficient to have any residual infectivity removed completely when stored at around 20 °C.
Much of the information on virus susceptibility by various routes to different animals is presented as 50% Tissue Culture Infective Dose ($TCID_{50}$). Due to the calculation of titres as pfu / ml, a conversion into $TCID_{50}$ is necessary in order to make a comparison possible. The following equation (Horzinek, 1985) being that the ID_{50} equals about 0.7 pfu or 1 pfu equals about 1.4 ID_{50} was deemed sufficient for this purpose.

Sellers (1971) reported on the experimental infection dose by oral route being about 10^4-10^5 $TCID_{50}$ for pigs and 10^5-10^6 $TCID_{50}$ for ruminants (see also review Alexandersen, 2003). The highest titre found in this study for untreated intestines was 1000 pfu or about 1400 $TCID_{50}$ per ml of homogenate. When fed to susceptible animals, several grams of untreated intestines may thus lead to an oral infection. Be that as it may, intestines processed into natural sausage casings are only traded and imported into the EU after salting and storage of at least 30 days, therefore meeting the requirements as laid down in current EU legislation.
Ample arguments can already be found to support salt-curing of casings as a valid virus-inactivating method, when reviewing studies done in the late sixties on the survivability of FMDV in animal products (Savi et al., 1962; Heidelbaugh and Graves, 1968; Cottral, 1969). However, these studies never focussed on intestines in particular and did not take into account the standard processing (cleaning and salting) and storage conditions (minimum period 30 days, room temperature) of casings.
The results described in **Chapter 5** show that casings originating from infected animals can be effectively treated to remove all FMDV infectivity.
Not only the treatment with phosphate supplemented salt but also the standard preservation treatment with NaCl and storage for 30 days at 20 °C is sufficient to remove FMDV infectivity. The results support that, in principle, the treatment laid down in Commission Decision 2004/414/EC (European Commission, 2004c) and Council Directive 2003/85/EC (European Commission, 2003) is effective. However, our results suggest that the storage temperature is of critical importance. A recommendation could therefore be made to have the temperature requirement entered together with the minimum storage period into specific legislation or measures to prevent the spread of FMDV.
As shown by the cited literature and the results from this study, the potential risk of FMDV transmission via salted casings can be reduced to an acceptable minimum without major changes to the common practises of the casing industry.

The recommendations based on the outcome of the FMDV inactivation study in **Chapter 5** were incorporated in the 2008 OIE Terrestrial Animal Health Code: *"For the inactivation of viruses present in casings of small ruminants and pigs, the following procedures should be used: salting for at least 30 days either with dry salt (NaCl) or with saturated brine (Aw < 0.80), or with phosphate salts / sodium chloride and kept at room temperature during this entire period".*
This clearly shows how industry related scientific research can be used to facilitate the international trade in casings when objective results are transformed into acceptable recommendations.

In addition to the described oral infection risk of FMDV via untreated intestines, CSF virus (CSFV) can spread via human food products derived from subclinically infected swine, when food waste is -illegally- fed to CSF-free domestic or feral pigs (Fritzemeier et al., 2000; Wooldridge et al., 2006).
For the infection model used in this study, a specific scenario was followed to mimic a plausible practical situation. A fattening pig was successfully infected with the highly virulent CSF virus strain "Koslov" (Mittelholzer et al., 2000; Wehrle et al., 2007) and killed during the prodromal phase of the infection, allowing it to pass slaughterhouse inspection undetected (Depner et al., 1997; Moennig et al., 2003). As a low dose of a highly virulent CSFV strain can lead to an infection (Dahle and Liess, 1992), all piglets could have become infected if fed

with a casings preparation that contained infective CSFV. Results from a similar infection model described by Helwig and Keast (1966), with CSFV by consumption of infected casings indicated that infection is possible, but that the virus survivability depended strongly on the processing and storage conditions used with the casings. This was further illustrated by Depner et al. (1998) who showed that CSFV can survive in casings when stored in saturated brine (pH 6.3) for a period of 30 days 4 °C.
As the survivability of CSFV reduces when the pH is either lowered or increased (Depner et al., 1992; Edwards, 2000) a likely inactivating agent would either be phosphate supplemented NaCl or citrate supplemented NaCl. Studies on the usability of citric, lactic and phosphate additives during the processing of casings showed a clear hygienic improvement by all three additives (Bakker et al., 1999), with a clear preference for phosphate due to a better handling during the stuffing process and improved tenderness of casings (Verkleij and Keizer, 2003; Houben et al., 2005; Nakae et al., 2008).
Although salted casings can be stored at 20 °°C without loss of quality (Bakker et al., 1999), lower storage temperatures (8-12 °C) are generally used by the casing industry (ENSCA, 2008). As a consequence, only the efficacy of the phosphate or citrate supplemented salt was determined at 4 and 20 °C to allow for any storage temperature over 4 °C.
Cleaned casings from the infected fattening pig were divided and treated with either agent and subsequently stored for 30 days at 4 or 20 °C. Casing suspensions produced from these different groups were fed to susceptible piglets and results show that only the treatment with citrate supplemented salt combined with storage at 4 °C was ineffective to inactivate the CSFV present, resulting in a clinical infection.
When combining the FMDV inactivation results described in **Chapter 5** (effective at 20 °C, using either salt or phosphate supplemented salt) and the CSFV inactivation results in **Chapter 6** (effective at 4 and 20 °C, using phosphate supplemented salt or using citrate supplemented salt at 20 °C) common ground is found when using phosphate supplemented salt at 20 °C. Combined with the industry's technical preference of phosphate over citrate and ambient storage temperatures, a recommendation on the inactivation of FMDV and CSFV in casings can be made to use phosphate supplemented salt during 30 days of storage, at a temperature of approximately 20 °C.

In order to determine that a treatment with phosphate supplemented salt has been applied for the inactivation of FMDV and CSFV in casings, an existing phosphate assay kit was validated for this product. **Chapter 7** describes the tests done on the evaluation of the Quantichrom™ phosphate assay kit (DIPI-01K, Gentaur Molecular products, bvba, Brussels, Belgium).
Results show that a qualitative analysis of the phosphate content of these casings is possible, with sufficient distinction of naturally occurring phosphate in salt and regardless of whether casings were preserved in dry-salt or brine. With a minimum of equipment (microplate reader, water bath and normal glassware) the phosphate content of casings can be determined within 3-4 hours. Overall, it can be concluded that the Quantichrom™ phosphate assay kit can be recommended as a standard method for phosphate analysis in treated casings, as a measure to prevent the spread of FMDV and CSFV.

The outcome of the CSFV inactivation study in **Chapter 6** will, as previously done with the FMDV inactivation study, be submitted to the OIE for a similar

evaluation and, combined with the validated method (**Chapter 7**) for phosphate analysis, may lead to the international recommendation for the prevention of contagious animal diseases via casings.

In conclusion, the results presented in the individual chapters provide guidance for quality improvement and control measures in the processing of natural sausage casings. In addition, some of the studies have affected recent legislative initiatives, particularly regarding safety measures taken to prevent the transmission of contagious animal diseases. The latter investigations need to be expanded to other transmissible diseases with the aim to ensure a free trade of casings. The microbiological safety of casings will remain an issue that requires continuous attention. Improvement of the preservation techniques remain to be explored aiming at a reduction of storage times and guaranteeing the absence of bacterial spores and other infectious agents in consumable products.

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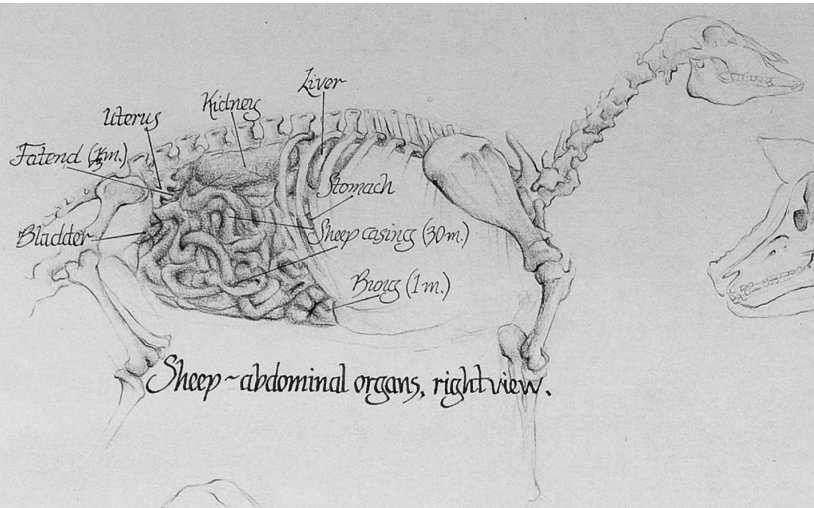
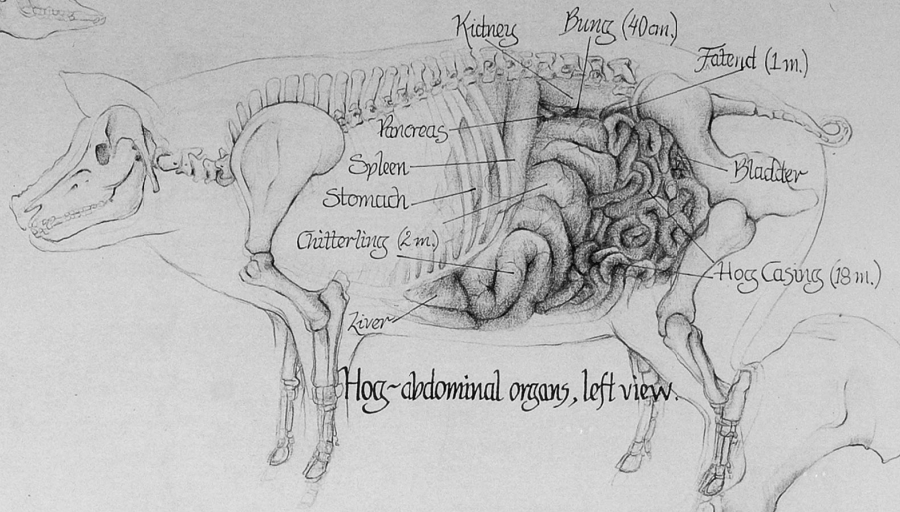
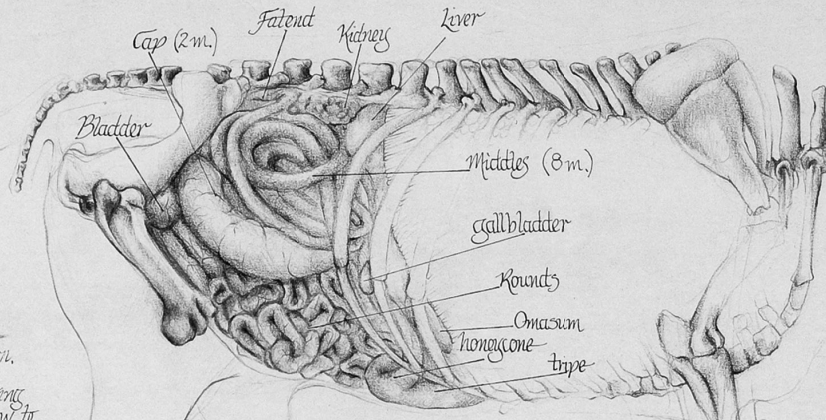


Sheep ~ abdominal organs, right view.



Hog ~ abdominal organs, left view.



Beef ~ view of abdominal organs from right

Four Steps to preparing casings for stuffing

1. Rinse salt from casings with fresh water.

2. Soften by soaking in fresh water at room temperature of 70° for 45 minutes to one hour. When hanks are placed in water, massage them with hands to separate the strands and to prevent dry spots which may occur when stuffing.

3. Take casings to stuffing table. Place in bath of fresh water. This water should be warmer (110°) to render a little of the natural fat in the casing. This will help the casing slide from the stuffing horn.

4. Preflush casings by introducing water into the casings and allow to run through the casing. This will facilitate getting the casing on the filling horn and moving the casing smoothly during the filling process.

Natural Casings

- allow deep smoke penetration
- stay tender and juicy during preparation
- protect the fine flavour of sausage
- curve and look great naturally
- are highly desirable
- contain no artificial ingredients

Storage Tips:

- Keep all fresh meat cold 32°-40° F.
- most sliced meat keep 4 to 7 days.
- Fresh and fully cooked sausage may be frozen up to 30 days
- When frozen sausage is thawed, use 2-3 days.
- Dry sausage may be kept for several months, unopened, in a cool place. Once casing is cut, treat as fresh.

Nederlandse samenvatting

Natuurdarmen worden geproduceerd uit de ingewanden van verschillende diersoorten zoals varkens, schapen en runderen en worden wereldwijd gebruikt als eetbare omhulsels van talloze worstsoorten.
Vanwege deze toepassing moeten natuurdarmen geschikt zijn voor humane consumptie en moeten zij voldoen aan de wettelijke eisen die gelden voor eetbare producten van dierlijke oorsprong.
Natuurdarmen zijn zeer nauw verbonden met worst ook al maken ze slechts 1-1.5% uit van het totale gewicht van een worst. Vanuit dit oogpunt is het dan ook begrijpelijk dat natuurdarmen zeer weinig wetenschappelijke aandacht hebben gekregen, simpelweg vanwege het feit dat bij een beoordeling van een worstje de aandacht uit gaat naar het totale product of het vlees wat in het worstje is verwerkt. Dit heeft tot gevolg dat er vele onderzoeken beschikbaar zijn voor worst, maar slechts een handjevol voor natuurdarmen.

Sinds 1 januari 2006 is er vernieuwde Europese wetgeving van kracht, waarbij de controle op hygiëne tijdens het productieproces volgens het HACCP principe plaatsvindt. Iedere productiestap dient volgens dit principe op mogelijke risico's geëvalueerd te worden en passende maatregelen dienen te worden genomen om de veiligheid van het product voor de consument te garanderen.
De hoofdstukken 2 en 3 gaan dieper in op de aanwezigheid van bacteriën in natuurdarmen tijdens het productieproces en bekijken op welke manier deze bacteriën in aantal teruggebracht kunnen worden tot een acceptabel niveau.
Alle bacteriën die een mogelijk risico vormen voor de volksgezondheid blijken door het conserveringsproces van schoongemaakte darmen met zout of volledig verzadigde pekel na 30 dagen opslag bij kamertemperatuur niet meer levensvatbaar. Deze manier van conserveren wordt al duizenden jaren toegepast tijdens de productie van natuurdarmen en met het onderzoek in hoofdstuk 2 is aangetoond hoe effectief zout als conserveermiddel is. Uitzondering op deze regel zijn de sporenvormende bacteriën. Deze kunnen zich als gevolg van de hoge zoutconcentratie omvormen tot spore en zijn zo in staat te overleven. Indien de omstandigheden weer gunstig zijn kunnen deze sporen opnieuw uitgroeien tot volwaardige bacteriën.
Hoofdstuk 3 beschrijft het onderzoek waarin een sporenvormende bacterie wordt blootgesteld aan verschillende concentraties nisine om zo uitgroei van spore tot bacterie tegen te gaan of te remmen. Nisine is een natuurlijk conserveermiddel wat al veel wordt toegepast in de zuivelindustrie. De resultaten van dit onderzoek laten zien dat uitgroei significant is afgenomen maar dat met nisine geen volledige remming of inactivering van sporenuitgroei mogelijk is.

Vanwege het mogelijke verband tussen BSE ("gekke koeienziekte") en de ziekte variant-Creutzfeld-Jakob bij de mens, die kan ontstaan door het eten van voedsel afkomstig van met BSE besmette runderen, zijn een aantal organen en karkasdelen van het rund uitgesloten voor humane consumptie. Deze uitgesloten groep wordt aangeduid als Specifiek Risico Materiaal (SRM) en ook runderdarmen worden hiertoe gerekend.
Hoofdstuk 4 beschrijft de weefselstudie die is uitgevoerd op runderdarmen afkomstig uit landen met een verwaarloosbaar BSE risico die wel in Europa geconsumeerd mogen worden. Uit deze studie komt naar voren dat een standaard worst van 200 g geproduceerd in runderdarm ongeveer 0.09 g lymfeweefsel en 0.02 g zenuwweefsel bevat, afkomstig uit de darm. Beide weefseltypen worden in verband gebracht met een mogelijke BSE besmetting.

Echter, tot op heden is er geen volledig sluitende onderbouwing dat deze weefsels in de runderdarm ook daadwerkelijk BSE bevatten.
Een aanvullende risicoanalyse laat zien dat het BSE risico voor de consument van een dergelijk worstje niet groter is dan van een T-bone steak, geproduceerd van dezelfde koe. Een T-bone steak bevat namelijk ook lymfe en zenuwweefsel maar wordt in Europa als een veilig product beschouwd. Deze bevindingen zouden mogelijk een aanzet kunnen geven tot een herziening van de Europese wetgeving op het gebied van BSE. Daarmee zou de productie van runderdarmen in Europa weer toegestaan kunnen worden.

De internationale handel in natuurdarmen wordt gereguleerd door strikte diergezondheids - en hygiëne voorwaarden, welke zijn weergeven op de gebruikte gezondheidscertificaten. Handelsbeperkingen kunnen worden ingesteld op basis van uitbraken van besmettelijke dierziekten. De enorme economische impact van dergelijke uitbraken kan worden geïllustreerd met twee recente Europese voorbeelden. De uitbraak van mond-en-klauwzeer (MKZ) in Engeland in 2001, totale kosten 8 miljard pond en de uitbraak van klassieke varkenspest (KVP) in Nederland in 1997, totale kosten 1.4 miljard euro.
Om het risico van een dierziekte-uitbraak als gevolg van de handel in natuurdarmen afdoende te beperken is dan ook specifiek onderzoek in darmen noodzakelijk. In de hoofdstukken 5 en 6 worden in twee studies het virusafdodend effect van zout en zout verrijkt met fosfaat beschreven. Hoofdstuk 5 laat zien dat het normale conserveringsproces van darmen met zout effectief een aanwezige besmetting met MKZ virus kan bestrijden, na opslag gedurende 30 dagen bij 20 °C. Ook het zout verrijkt met fosfaat is hiervoor geschikt.
De aanbevelingen uit dit hoofdstuk zijn inmiddels overgenomen door de Wereldorganisatie voor Diergezondheid (OIE) in de Terrestrial Animal Health Code, versie 2008.
Hoofdstuk 6 laat zien dat alleen het zout verrijkt met fosfaat in staat is om KVP virus te inactiveren na 30 dagen opslag. In dit geval speelt de temperatuur tijdens opslag een minder belangrijke rol.
Indien de resultaten van beide hoofdstukken worden samengevoegd kan een aanbeveling worden gedaan om natuurdarmen gedurende 30 dagen bij 20 °C op te slaan in zout verrijkt met fosfaat om zo een MKZ of KVP besmetting te inactiveren.
Om aan te kunnen tonen dat natuurdarmen daadwerkelijk zijn behandeld met zout verrijkt met fosfaat, is een bestaande fosfaatanalysemethode gevalideerd voor dit onderzoek in natuurdarmen. Hoofdstuk 7 beschrijft dit onderzoek en toont aan dat deze analysemethode geschikt is voor dit doel.

Ter conclusie, de resultaten welke zijn gepresenteerd in de verschillende hoofdstukken bieden de nodige handvatten voor de kwaliteitsbewaking tijdens de productie van natuurdarmen. Daarbij hebben aanbevelingen gebaseerd op deze studies inmiddels geleid tot aanpassingen van de internationale richtlijnen ter preventie van de verspreiding van besmettelijke dierziekten. Andere besmettelijke dierziekten zullen op vergelijkbare wijze onderzocht worden en waar mogelijk zullen de aanbevelingen uit deze studies bijdragen tot een vrij handelsverkeer van natuurdarmen. Het microbiologisch onderzoek van natuurdarmen zal een aandachtspunt blijven in vervolgstudies, waarbij specifiek zal worden gekeken naar verbetering van de conserveringmethodes om de afwezigheid van bacteriën en bacteriesporen in voedselproducten te borgen.

Ook al staat er maar één naam op de kaft van dit proefschrift, er heeft toch een heel team van mensen aan bijgedragen. Aan jullie allen, heel hartelijk dank voor alle hulp en inspanning.

Hooggeleerde professor van Knapen, beste Frans, mijn 1e promotor. Toen jij in 2005 werd benaderd was jouw enige voorwaarde een duidelijk plan voor dit proefschrift, daar ik dit zou gaan doen in combinatie met mijn werk bij Van Hessen. In ruil hiervoor bood jij de mogelijkheid om nauw samen te werken met de stafleden van jouw afdeling met als resultaat een aantal goede studies welke een groot deel uitmaken van dit proefschrift. Hartelijk dank hiervoor.

Hooggeleerde professor Fink, beste Johanna, mijn 2e promotor. Al in de periode 2000-2003 heb je me geholpen bij een eerdere poging om te kunnen promoveren. Daarbij heb je ook een heel belangrijke rol gespeeld om de overstap te maken van de praktische diergeneeskunde naar de veterinaire volksgezondheid en een baan in de natuurdarmindustrie. Vanaf 2004 heb je als mijn mentor gefunctioneerd voor mijn werk binnen de industrie en zijn we er in geslaagd om in 2005 een gedegen plan op tafel te leggen voor een proefschrift met een praktische toepassing. Jouw inspanningen en vertrouwen zijn me bijzonder veel waard en ik hoop dat we in de toekomst op deze wijze kunnen blijven samenwerken.

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Lex, als ik jouw bijdrage aan mijn promotie volledig zou beschrijven zou ik een extra hoofdstuk aan dit proefschrift hebben kunnen toevoegen. Op voorzet van Johanna heb je mij in 2004 aangenomen binnen Van Hessen en mij gedurende het eerste jaar alle ruimte gegeven om de industrie te leren kennen. De afgelopen jaren hebben we op velerlei vlakken succesvol samengewerkt en heb je duidelijk aangeven wat de waarde is van wetenschappelijk onderzoek voor de natuurdarmindustrie. Zonder jouw steun was dit proefschrift helemaal niet mogelijk geweest en daarbij ben je een goede motivator geweest om zaken op tijd af te krijgen. *"Toda raba".*

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Last but not least, mijn paranimfen Jaap en Jan-Willem, hartelijk dank voor al jullie steun en hulp.

Joris Jan Wijnker was born on October 1st 1967 in Aarle-Rixtel. He attended the Carolus Borromeus College and passed his final exams in 1986. He started his studies of veterinary medicine at Utrecht University that same year. These studies were interrupted by serving 18 months in the army medical corps in 1989 and 1990. In November 1998 he graduated *with honours* at Utrecht University and started as general practitioner in a private clinic for companion animals specialised in exotics. In December 1999 he returned to the Faculty of Veterinary Medicine in Utrecht, at the Department of Equine Sciences, Section of Equine Anaesthesiology. Having finished his 4 year contract he started as Veterinary Advisor for Van Hessen bv in January 2004. This position offered the possibility to start his PhD track at the Department of Veterinary Pharmacology, Pharmacy and Toxicology (VFFT) and the Division Veterinary Public Health of IRAS in May 2005. He is currently working as Chief Veterinary Officer at Van Hessen bv, as Executive Secretary of the European Natural Sausage Casings Association (ENSCA) and Scientific Director of the International Scientific Working Group (ISWG) of the international casing industry.

Joris Jan Wijnker werd geboren op 1 oktober 1967 in Aarle-Rixtel. In 1986 werd het eindexamen VWO behaald aan het Carolus Borromeus College in Helmond. Dat zelfde jaar is hij begonnen met de studie Diergeneeskunde aan de Universiteit Utrecht. Deze studie is onderbroken voor 18 maanden militaire dienst bij het Korps Geneeskundige Troepen in 1989 en 1990. In november 1998 legde hij het dierenartsenexamen *met genoegen* af en begon als gezelschapsdierenpracticus in een kliniek toegelegd op exotische diersoorten. In december 1999 keerde hij terug naar de Faculteit Diergeneeskunde, bij het Departement Gezondheidszorg Paard, Sectie Anesthesiologie. Na afronding van zijn 4 jarig contract is hij in januari 2004 begonnen als Veterinair Adviseur voor Van Hessen bv. Deze positie bood hem de mogelijkheid om te gaan promoveren bij de Afdeling Veterinaire Farmacologie, Farmacie en Toxicologie (VFFT) en de Divisie Veterinaire Volksgezondheid van het IRAS in mei 2005. Op dit moment werkt hij als Chief Veterinary Officer bij Van Hessen bv, als Executive Secretary van de European Natural Sausage Casings Association (ENSCA) en als Scientific Director van de International Scientific Working Group (ISWG) van de internationale natuurdarmindustrie.

ISWG

International Scientific Working Group