Effects of biological factors on collagen quality of livestock animals for producing co-extruded sausage casings

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Effects of biological factors on collagen quality of livestock animals

for producing co-extruded sausage casings

Patricia Suurs

Propositions belonging to the thesis, entitled

Effects of biological factors on collagen quality of livestock animals for producing co-extruded sausage casings

1. Pre-processing conditions strongly affect collagen quality for co-extrusion sausage casings.
   (this thesis)

2. Sausage casings produced with collagen dispersions obtained from porcine urinary bladder/ intestine result in the best sausages.
   (this thesis)

3. Music is necessary for the development of the young child brain.

4. Insect breeders are the farmers of the future.

5. "Plus classes" only for gifted children in elementary schools lead to social inequality between pupils.

6. Parenting ensures a good balance between PhD-study, work and family life.

Patricia Suurs
Wageningen, 14 June 2023

Effects of biological factors on collagen quality of livestock animals

for producing co-extruded sausage casings

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Effects of biological factors on collagen quality of livestock animals
for producing co-extruded sausage casings

Patricia Rosa Maria Suurs

Thesis

Submitted in fulfilment of the requirement of the degree of doctor
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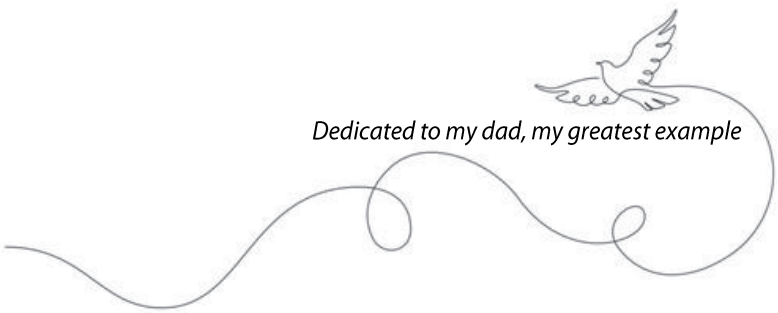

Dedicated to my dad, my greatest example

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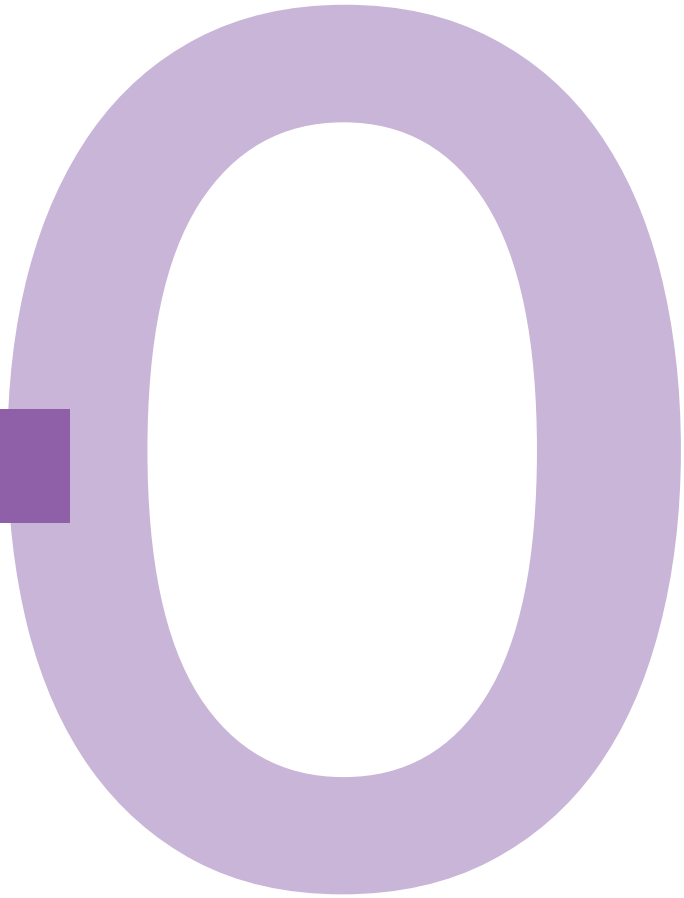
CHAPTER 0



General introduction

1. Sausage casings

Sausages represent one of the oldest types of processed meat products, where a meat mass together with salt, water, seasonings (herbs/spices) and binders are stuffed into natural or manufactured casings. Casings are thin tubular skins or films of different single/multilayer material(s), enclosing the sausage meat batter. The casings contribute to the mechanical stability and other functional properties of the sausage. For example, collagen casings contain long fibers, embedded in a highly organized matrix and this composite structure possesses unique characteristics of high strength and low elongation; two characteristics that are important in casing functionality (Savic and Savic, 2016). Casings based on natural biopolymers can be classified into two categories: 1) natural casings derived from animal tissue (intestines), that maintain the original natural connective tissue conformations and only have limited macromolecular structural changes during their conversion into sausage casings, 2) manufactured casings made from collagen, cellulose, alginate or their combinations. They are derived from their modified natural structure, which are extracted from, for example animal or plant tissues (Savic and Savic, 2016). Casings made from actual intestines, collagen and cellulose are mainly used by sausage manufacturers (Harper, 2013).

This general introduction is meant to introduce several aspects of sausage casings. Paragraph 2 describes the different casing types (e.g., natural casings and (processed) manufactured casings), followed by casing functionality in paragraph 3. Paragraph 4 will then describe the collagen structure, after which Paragraph 5 focusses on the co-extrusion process and bovine skin collagen, which is currently used as a raw material for making a casing in the co-extrusion process. Paragraph 6 discusses alternative collagen sources as raw material for casing production in the co-extrusion process, followed by paragraph 7, discussing the factors influencing the collagen quality of co-extruded sausage casings and the associated knowledge gaps. Paragraph 8 finally describes the aim of the thesis, followed by the outline of the thesis.

2. Types of casings

2.1 Natural casings

Traditionally, sausage meat has been stuffed into intestines derived from pigs, sheep, and cattle (Wijnker, 2009). They are by-products of the livestock industry (Savic and Savic, 2016). Ovine and porcine casings are made from the small intestines. The small intestine consists of 5 layers, including the submucosa. The submucosa layer is predominantly comprised of a dense connective tissue layer, containing type I and III collagen

in the form of cylindrical sheets and elastic fibers, providing strength and elasticity to the gut wall (Gunn *et al.*, 2022). For the casing production, the submucosa is obtained after washing and scraping the other intestinal layers (Koolmees *et al.*, 2004), followed by soaking in ice water or brine to remove blood, after which grading is performed according to type, size, and quality. The last process step is curing in salt for preservation purposes by lowering the water activity (Koolmees *et al.*, 2004).

Natural casings are considered the golden standard for sausage casings by the industry as consumers value their favorable organoleptic properties. Natural casings exhibit special characteristics, such as elasticity, good appearance (curvature), tenderness, a good "knack" or snap, and permeability, which allows smoking flavors to diffuse through the casing into the meat, thereby enhancing the flavor of the sausage (Harper *et al.*, 2012; Gunn *et al.*, 2022). The disadvantages of natural casings are the lack of consistency in diameter, length, thickness, and potential weak spots (Gunn *et al.*, 2022; Savic and Savic, 2016). Moreover, natural casings are relatively expensive, exacerbated by supply issues caused by African Swine Fever (Anonymous, 2021). In addition, working with natural casing involves more labor in their cleaning and application. Today, natural casings are used for selected products to provide a traditional look and a certain "snap" (Barbut, 2015).

Figure 1 shows how the global market has developed regarding manufactured collagen casings and natural casings. In 2008, the natural casings comprised 70% of the market, in 2021 this was only 51%, with the manufactured collagen casing gaining a larger share of the global market (Anonymous, 2021). In 2020, the shift from natural intestines to collagen casings was enhanced due to the Covid pandemic and the impact on the global labor markets. Labor shortages and high prices for intestines are causing sausage producers to re-evaluate their casing strategy (Anonymous, 2021).

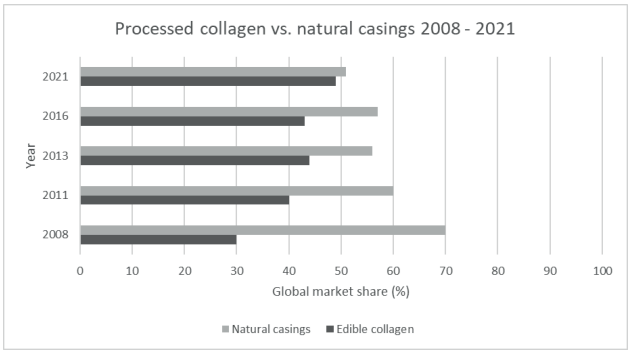


Figure 1. Global collagen market share of processed collagen casings versus natural casing in period of 2008 – 2021 estimated by Devro plc, reflecting the sausage market growth of intestine to processed collagen conversion (Anonymous, 2019; 2021). NB. other edible casings, such as cellulose and alginate are not included in the figure as the focus of the thesis is on processed collagen casings.

2.2 Processed (manufactured) casings

Due to the relatively high price of natural casings and their limited availability, the sausage casing industry has been searching for alternatives. Already in 1960, the first edible manufactured collagen casings appeared on the market (Savic and Savic, 2016). Nowadays, processed casings from organic materials include casings made from cellulose, alginate, collagen, and combinations of both, also known as hybrid casings. Cellulose casings are non-digestible and must be peeled off prior to consumption. They are permeable to water and smoke and are produced on highly automated equipment and are very popular for high volume products, such as Frankfurter type sausage (Savic and Savic, 2016; Barbut, 2015). For small diameter products, like hot dogs, the casings are peeled off at the plant with an automated high-speed peeler. For larger diameter products, such as salami, the casing is more commonly peeled off by the consumer just prior to consumption (Barbut, 2015). Three types of cellulose casings can be distinguished: 1) non-fibrous cellulose casings, 2) fibrous cellulose casings and 3) plastic coated fibrous cellulose casings (e.g., with polyvinylidene chloride; PVDC). The non-fibrous and plastic-coated cellulose casings are mainly used to produce Wieners, hot dogs, and smoked sausages and are removed at the plant after final processing. Fibrous cellulose casings are mainly used to produce dry sausages.

Alginate refers to a group of naturally occurring polysaccharides that are extracted from the brown seaweed (*Phaephycea)*. Alginate constitutes of two monosaccharides, guluronic and mannuronic acid and it can form cold-set gels in the presence of polyvalent metal cations (most commonly calcium) (Harper, 2013). Depending on the ratio of guluronic and mannuronic acid, strong or more flexible casings can be formed. It should be mentioned that the proportion of guluronic and mannuronic acid residues will vary depending on the algal source (Fang *et al.*, 2007). The ability to form cold-set gels makes that alginate can form a casing without a drying and/or heating step. In the process, a thin layer of alginate gel, submerged in a calcium chloride solution, forms a casing which can be applied immediately on the food product e.g., meat batter. This type of casing is suitable for producing fresh sausages (such as chipolata), breakfast sausages (individually quick frozen) and vegetarian sausages. For sausage producers, one of the challenges of this type of casing is to maintain a stable Ca-alginate casing during shelf life of the sausage (Visser, 2012). Leaching of Ca^{2+} from the casing to the meat batter can result in the disintegration of the Ca-alginate casing to form a sticky Na-alginate gel on the sausage surface. Sausage manufactures can add a calcium source, such as calcium lactate, to the meat batter formulation to balance the Ca^{2+} gradient (Harper, 2013). However, sausage manufacturers are often hesitant to adjust their meat batter formulations.

Next to pure alginate casings, hybrid casings made of, for example, collagen and alginate are used. These casing types are first gelled with calcium chloride solution followed by air-drying and optionally crosslinked with liquid smoke (Harper, 2013). The development of this casing type of casing started due to the shelf-life issues of alginate casings. The presence of collagen in the dispersion improves the stability of the casing. Such hybrid casings are suitable to produce fresh sausages with a maximal lifespan between 10 and 14 days (Bontjer *et al.*, 2014).

Manufactured collagen casings are developed as an alternative for natural casings. Manufactured collagen casings have become more popular as they became edible, and their organoleptic properties are perceived by consumers close to natural ones. A major advantage is being less prone to microbial contamination, longer shelf life, their uniformity in size, length, and diameter, having high mechanical strength and their thermal stability. They are less expensive and easier to work with compared to natural casings as they can stand higher filling pressures and require less labor (the casings arrive as a tube that can be put directly onto the stuffing horn). Moreover, manufactured collagen casings do not have weak spots (Sobanwa, 2021; Savic and Savic, 2016) that in case of natural casing require stopping the operation. Both manufactured and natural casings have a finite length, which makes that sausage production remains a batch operation. As shown in Figure 1, it is estimated that the global usage of collagen casings for sausage production increased from 30% in 2008 to 49% in 2021 (Anonymous, 2019, 2021). Both, natural casings and processed casings, play important functional roles in the sausage manufacturing process from the moment of stuffing right up until the consumer eats the product.

3. Casing functionalities

The selection of the correct casing is critical, as it is not only influencing the integrity, size, and shape of the sausage, but also assists in the conversion of the soft/semi solid meat batter into the desired cooked/dried sausage texture (Savic & Savic, 2016). The ideal collagen sausage casing should provide sufficient strength to undergo processing, while remaining tender enough during consumption (Miller, 1983). It is not possible to create an all-purpose sausage casing suited for all types of sausages, as processing requirements can greatly vary. This thesis focusses on casings to produce cooked smoked sausages with collagen as raw material. From an industrial perspective, the most important properties of casings are their mechanical strength (e.g., tensile strength, elasticity), their barrier properties (e.g., permeability to water vapor, smoke, and gases), and thermal properties (Sobanwa, 2021).

Harper *et al.* (2012) demonstrated that a wide variety of manufactured casings with different mechanical properties do exist on the market. The mechanical strength is important, because casings must be strong enough to hold the meat but must also be able to expand (elastic properties) by a small percentage during stuffing and cooking (Bakker *et al.* 1999; Simelane and Ustunol, 2005). Additionally, the casing also helps to control the meat proteins gelling process of the meat batter, which influences the final structure of the sausage matrix. The mechanical strength is also important as it influences the consumer's perception of toughness (or bite/snap) of the final product (Bakker *et al.*, 1999). Overall, the elasticity and tensile strength of the casing are important parameters, as they affect sausage final diameter, shape, structure, behavior during processing, and bite of the final product (Sobanwa, 2021).

Permeability of the casing is another important factor, because the casing needs to be permeable for water vapor, smoke, and gases. The water migration, and for example, liquid smoke into and out of the sausage is important as it contributes to the texture, nutritive and marketing value of sausages. The migration of these elements through the casing is determined by concentration gradients and the microstructure of the casing, whereby the polarity of a material is also an important factor. Protein films have high permeability to polar substances, such as water vapor, and low permeability to non-polar substances, such as oxygen, aroma compounds and oils. The permeability depends on the type of casing, their moisture content, pH, and water activity of the meat batter (Krochta, 2002; Savic and Savic, 2016). Collagen, for example, is a relatively good oxygen barrier, but not a good moisture barrier (Krochta, 2002; Savic and Savic, 2016). Oxygen permeability is of importance for shelf-life considerations, where casing type and thickness are determining factors. The presence of oxygen in the sausage matrix affects fat oxidation rate and color changes (Savic and Savic, 2016). In fact, some casings can be used as a barrier to moisture and other substances that cause quality deterioration of the sausage.

Thermal properties of a casing are important for sausage types that are subjected to treatments, such as pasteurization, sterilization, grilling and roasting. The casing should be resistant to high temperatures, thereby keeping the round shape of the sausage and protect the meat batter from unwanted changes and influences (e.g., yield losses) (Savic and Savic, 2016).

4. Collagen structure

In the animal body, collagen is a structural protein, present in the extracellular matrix (ECM) and connective tissue of animals, which makes up approximately 30% of the

entire animal body protein content (Silvipriya *et al.*, 2015; Sobanwa *et al.*, 2020). One of the functions of collagen in the animal body is to provide support to tissues, both structurally and mechanically. So far, more than 28 distinct types of collagens have been described (Sionkowska, 2021; Liu *et al.*, 2019). Collagens can be divided into two major groups: fibrillar and non-fibrillar (Maxwell, 2007). Fibrillar collagen types, such as type I and III collagen, are found among others in skin, tendon, blood vessels (Shoulders and Raines, 2009). Among all collagens, fibrillar collagens account for about 80 – 90% of the mammalian body's collagen (Xiong, 2008; Silvipriya *et al.*, 2015; Sobanwa, 2021).

Type I collagen molecules are composed of three α-chains encoded by the COL1A1 and COL1A2 gene, thus forming heterotrimers. The consensus sequence $(Gly\text{-}X\text{-}Y)_n$ in collagen rise to these α-chains (Figure 2). The X and Y can be any amino acid, but proline and hydroxyproline frequently occupy the X and Y position, respectively. The presence of proline and hydroxyproline force the peptide chain into a left-handed helix. They are twisted together to form a right-handed triple helix. The triple helix is the basic molecular unit of collagen and has a molecular weight of approximately 300 kDa, repeated unit length of 280 nm and width of 1.5 nm (Brodsky *et al.*, 2003; Gelse *et al.*, 2003; Xiong, 2008) and is stabilized by hydrogen bonds and Van der Waals forces. Figure 2 shows a detailed picture of the synthesis and collagen structure, starting from tropocollagen (collagen molecule) to collagen microfibril, collagen fibril and collagen fiber (Hoogenkamp *et al*., 2017).

The N- and C-terminal ends of the three α-chains are called telopeptide regions, which are characterized by a lack of triple helical structure. The telopeptide regions are rich in lysine and hydroxylysine residues. They can crosslink with other extracellular matrix proteins after aldehyde formation under the influence of the enzyme lysyl oxidase and stabilize the molecule and surrounding molecules via intra-/intermolecular crosslinking. Collagen molecules overlap approximately one-fourth with its neighboring molecule (67 nm) due to assembling in a quarter-staggered fibril array (Chambers, 2004; Shoulders and Raines, 2009; Yang et al, 2019). Until a fibril of a certain dimension is formed, the process of self-assembly will continue. Transmission electron microscopy shows a characteristic banding pattern of alternating light and dark striation because of alignment of collagen molecules (Shoulders and Raines, 2009). In the food industry collagen applications, most of the time, need alterations of the native collagen structure by for example chemicals to break some of the crosslinks and make the collagen more soluble. This is done with the acid swollen collagen, consisting of both insoluble and soluble collagen, which is used to produce co-extruded collagen casings (Sobanwa, 2020).

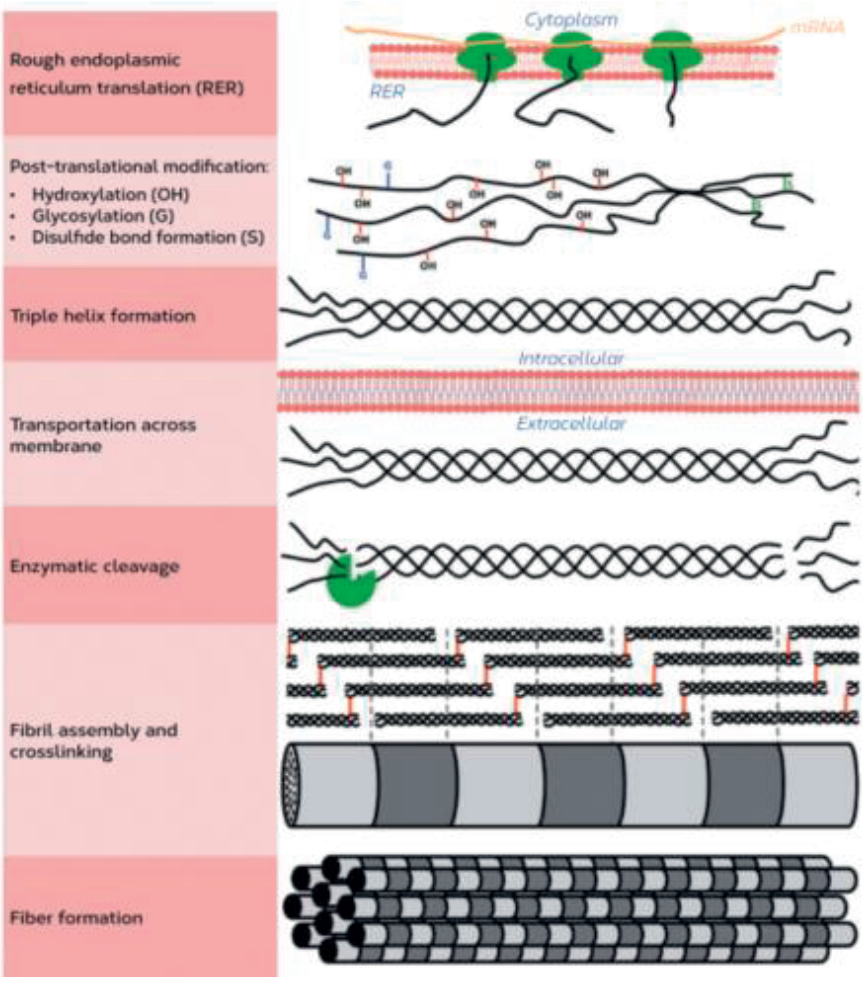


Figure. 2. Cartoonized scheme of the synthesis and structure of collagen (reproduced from Hoogenkamp *et al.*, 2017 with permission).

5. Co-extrusion

Initial reports and patents about co-extrusion showed that the technology dates back 40 years ago. However, due to technical challenges, the co-extrusion technology was not widely adopted until 10 – 15 years ago. Problems with casing stability as well as high investment cost and complexity of the process made the sausage manufacturers reluctant to implement the new technology (Savic and Savic, 2016). Townsend Engineering significantly improved the technology in the late 1990's and within the last 15 years, several major North American and European companies adopted the technology on a large scale (Escoubas *et al.*, 2010).

Today, the sausage industry is characterized by two main processes to stuff and cook products: 1) the traditional and most commonly used, semi-continuous operation, whereby meat batter is stuffed in the casing, followed by loading the sausages on sticks, which later transfers the sausages into a smokehouse to be smoked and cooked, 2) fully automatous sausage production that uses co-extrusion technology, where the casings are formed on the spot, as the sausage is being produced. The main feature of co-extrusion technology is the simultaneous extrusion of a thin layer of semi fluid collagen material onto a continuous flow of meat batter (emulsion or coarse ground), eliminating the intermediate stages of preparing and storing pre-made casings as is done in natural and/or manufactured casings (Savic and Savic, 2016). The co-extrusion process consists of several steps that can be divided into five zones (Figure 3).

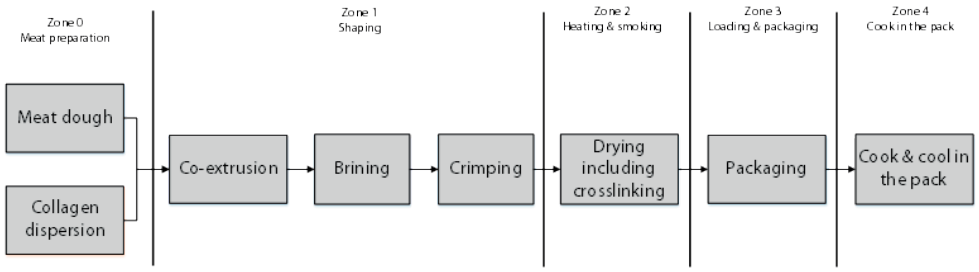


Figure 3. Schematic overview of the co-extrusion process for the preparation of cooked smoked sausages, indicating the different zones. Picture courtesy of Marel.

Zone 0 is related to receiving the raw collagen dispersion from the manufacturer and the meat preparation. Good meat preparation, especially for coarse ground and emulsified sausages is important as this can prevent quality issues, such as fat exudation. Fat exudation from the meat matrix can result in fat pockets on the sausage surface, which leads to poor smoke absorption. The collagen dispersion should be cold and free of any microbiological contaminants. Zone 1 is related to shaping of the sausage, creating a casing on the spot as the sausage is being produced. In this step, counter rotating cones are being used to steer the collagen fibers into the desired direction. The collagen travels in between the inner and outer cone, followed by extrusion through a 350 µm circular die, forming a casing. By applying different inner and outer cone speeds, the collagen fibers can be oriented in a more criss-cross or longitudinal direction, depending on the customers' needs (Hoogenkamp *et al.*, 2015). After extrusion, the sausage rope is submerged in a brine solution (saturated salt). The brine ensures that the proteins in the dispersion precipitate, lowering the water content of the casing by osmosis. This gives the sausage, including the casing, its initial strength for further processing. The final step in Zone 1 is crimping, cutting the endless sausage rope into separate sausage links. After crimping, the sausages enter Zone 2, where the meat batter and the casing

are further stabilized by air-drying and crosslinking with smoke condensates (Kobussen *et al.*, 2000). This zone utilizes a pre-drying step, followed by liquid smoke treatment and a post-drying step. In the pre-drying step, the coagulation of the meat batter is initiated, thereby further stabilizing the meat batter. The internal core temperature of the sausages is about 50 – 55°C at this point. Moreover, the casing is dried, meaning that the smoke condensate can be absorbed, and crosslinks can be formed. Aldehydes present in the smoke condensate are reacting to form covalent crosslinks in the casing (Toledo, 2007), which enhances the mechanical properties (Morgan *et al.*, 1988; Bontjer *et al.*, 2011; Zhang *et al.*, 2022). Next, the sausage surface is dried, during which covalent crosslinking continues. The internal temperature of the sausages, when leaving the dryer is approximately 55°C. In Zone 3, the sausages are automatically loaded into packages, which significantly helps to reduce labor costs compared to the stuffed casing production process. In Zone 4, vacuum packed sausages are cooked in water to an internal core temperature of ³ 74°C and rapidly cooled down to a core temperature of £ 15°C to obtain a safe product (Savic and Savic, 2016). The cook-in-bag principle prevents the risk of post-cooking microbial contamination (Harper, 2013).

The major advantage of the co-extrusion technology is that it allows manufacturers to move to a continuous operation as opposed to the traditional batch process and therefore enables large production volumes over 24 h/day (Smits, 1985). Although initial investment in the equipment is high, the increased output, reduced labor costs, uniformity of the outcoming products and lower risk of microbial contamination makes the technology very appealing to meat processors (Bontjer *et al.*, 2011). Additionally, the amount of waste and re-work are significantly reduced when using the co-extrusion principle (Anonymous, 2012).

5.1 Bovine skin collagen

To produce a collagen casing with co-extrusion technology, a collagen dispersion prepared from bovine skins is the most used method today on the market. The collagen is a by-product of the meat packing industry (e.g., beef cattle, skins that first go to the leather tanneries, where the grain is separated from the collagen rich corium layer). The bovine skin is composed of three principal layers: the outer layer or the epidermis and the structure below it, known as the grain and corium/dermis (Figure 4). The epidermis and dermis are separated by the basement membrane. Below the dermis is the hypodermis or subcutis, consisting of subcutaneous fat. The corium/dermis is the layer under the epidermis, providing physical strength and flexibility to skin. The corium/dermis is divided into two regions: the papillary dermis, which lies immediately beneath the epidermis and the deeper reticular dermis. The papillary dermis is composed of loose connective tissue containing epidermal structures like hair follicles, and sebaceous glands and form papillae that connect to the epidermis. The reticular dermis

has a denser meshwork of thicker collagen than the papillary dermis and contains large bundles of collagen and elastic fibers interwoven in many directions. It provides the skin with most of its strength, flexibility and elasticity. The corium/dermis thickness varies in different anatomical regions of an animal. Fibroblasts, which are scattered throughout the corium, are the cells responsible for the synthesis of collagen and elastic fibres (Falanga, *et al.* 2014; Savic and Savic, 2016).

Figure 4. Overview of the different layers in cattle skin used for leather production. Picture courtesy of Dbramante1928.

The reticular dermis is used to produce second-grade leather (suede) or collagen dispersions for the food industry, while the papillary dermis is used for (full grain) leather production. Full grain is the highest quality leather in its natural form without alteration. To remove the hairs/fur and to cut the top grain from the split (Figure 5) the skins are soaked in a lime solution at the tanneries.

Then the collagen rich corium layer (reticular dermis) is shipped to collagen manufacturers were the extraction process starts with deliming of the skin to remove impurities by addition of alkali, followed by acid addition to reduce the pH value to near the isoelectric point; for bovine collagen this is approximately 4.8. Next is washing with water and grinding the skins into small particle fibers that are dispersed in water to form a pulp. Finally, acid is added (acetic or hydrochloric acid) to induce fiber swelling, after which homogenization results in a well-mixed acid-swollen collagen dispersion (Sobanwa, 2021; Matinong *et al.*, 2022).

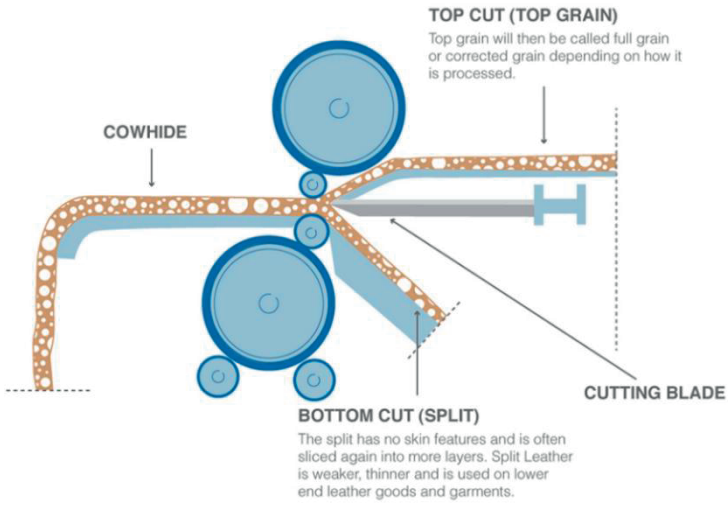


Figure 5. Overview how the cowskin is separated in top grain and split at the tanneries by a Splitter (leather splitting machine). Picture courtesy of Tusting.

6. Alternative collagen sources

There is an abundant availability of collagen in meat processing plants from which approximately 80% of the collagen and its derivatives are used of which ~32% in the food and beverage and ~48% in the health management industries in 2020 (Cao, Xiao, Ge, & Wu, 2021). These numbers show that the proportion of collagen in animal by-products used in the food industry is low (~32%), so there is potential to search for more collagen applications in the food industry. Furthermore, ~20% of the collagen and its derivatives are unutilized, which could lead to higher disposal costs and the loss of potential revenues (Tang *et al.*, 2022). The sausage industry predominantly uses fibrous type I collagen from bovine skins for their sausage casings. However, due to diseases, such as bovine spongiform encephalopathy, transmissible spongiform encephalopathy, food and mouth disease, the food industry is searching for alternative collagen sources (Silvipriya *et al*, 2015; Oechsle, 2016; Sobanwa, 2021). Moreover, as meat products have increased in price over the past two decades, a shortage of natural casings together with a significant price increase put even more pressure on the search for alternative collagen sources for sausage casing production (Harper *et al.,* 2012; Oechsle, 2016). Collagen can be extracted from mammals (e.g., cattle, pigs), marine invertebrates and vertebrates (e.g., jellyfish, starfish, cuttlefish) and poultry (e.g., turkey, chicken). Different species are likely to influence the quality of the collagen dispersion, which will be discussed in paragraph 7.1.

7. Factors influencing collagen quality

The collagen structure can be influenced by animal and environmental factors, such as age, housing, feed, etc. (Hood, 1987; Covington, 2011; Savic and Savic, 2016). However, it is unknown what the influence of these factors is on the collagen quality for the co-extrusion application. In the following section, potential factors affecting the collagen quality of livestock animals are described in greater detail as well as the associated knowlegde gaps.

7.1 Species

Today, bovine skin is predominantly used as a collagen source to produce dispersions for co-extrusion applications. In the past, there have been developments to use porcine skin as a source of collagen for dispersions (Visser, 2021). However, porcine skin collagen was found to have more challenges compared to bovine skin collagen, especially regarding the collagen extrusion process. The pork raw collagen material differs from bovine collagen, meaning that the fiber structure is weaker and the pork skin contains much more fat than beef skin. In pork this fat is integral to the collagen structure rather than existing as a separate layer as it is in beef skin. So, to prepare the porcine skin as a collagen dispersion, the major part of this fat needs to be removed, without damaging the collagen structure too much (Savic and Savic, 2016). For this reason the focus of this thesis was not on porcine skin, but on other porcine collagen sources, such as the intestine and urinary bladder. Today, no research data are available on the use of these sources in the production of collagen dispersions for co-extrusion purposes.

In addition to cattleskin, a poultry collagen dispersion is currently commercially available on the market (Anonymous, 2021). However, the amount of information regarding the performance of the gel on the co-extrusion line is still very limited. Information about e.g., extrusion and mechanical properties is lacking on dispersion specifications. Investigating the properties of poultry dispersions in relation to the co-extrusion process, could give sausage manufacturers more insight in the properties of the dispersion and give them tools to possibly adjust the process in case of production problems (e.g., gelatinized casing) or to solve quality problems of end products. Oechsle *et al.* (2016) was the first to investigate the microstructure and some physical-chemical properties of chicken skin collagen as a source of collagen for the co-extrusion process. However, in their research no clear relationships were established between the properties of the collagen and the required properties for the co-extrusion process, such as thermal stability and mechanical strength. Overall, it is necessary to understand the structure and properties of the potential collagen sources related to the co-extrusion process to fully exploit the application potential of alternative collagen sources in the sausage (casing) industry.

Marine collagen has gained more interest over the years (Silvipriya *et al.*, 2015) as it has several advantages over collagen from land animals, such as high collagen content, free of zoonosis, less ethical and religious constrains. However, the disadvantage of marine collagen is the low thermal stability, which limits its industrial applications (Sobanwa, 2021) and therefore is not part of the thesis.

Finally, no scientific data are available to compare the collagen quality of the various animal species for the co-extrusion application. Every animal species (e.g. cattle, pork, poultry) has its own specific live animal housing, environmental conditions and life cycle, etc., which determines the pretreatment process and the final properties of the collagen.

7.2 Breed/Strain

To produce commercial bovine collagen dispersion, the skins' corium of animals aged 18 – 36 months is being used. These animals are castrated males and in the industry their skin is referred to in the industry as ox (Europe) or steer skins (USA) (Visser, 2021). The question rises why other cattle skins, and specifically the skins of Dutch cattle, are not used to produce collagen dispersion for co-extrusion. Especially within cattle, differences exist in breed and animals' final purpose, i.e., kept for meat production (calves), breeding/milk production (cows), or as working animals (ox) (Noorzai *et al.,* 2019). Animals kept for meat production are relatively young at slaughter age (6 – 8 months), whereas animals kept for breeding/milk are much older, resulting in differences in crosslinked skin collagen (higher with increased age). From practical experience, it is known that it is more difficult to prepare a good extrudable gel from bull (intact males) skins than from steer (ox) skins (Visser, 2021). However, no scientific data is available showing why collagen producers favor the steer/ox skins to produce dispersions for co-extrusion applications. Additionally, no scientific data is present showing the differences in collagen dispersion quality when using other cattle skins as collagen source for co-extrusion. Considering that the leather industry produces skin trimmings from a variety of cattle breeds that some end up in landfill, it is interesting to investigate whether collagen extractions from these trimmings can be used to increase their value (Noorzai *et al*., 2019; Matinong *et al*., 2022).

In chicken processing operations, there are also different strains, such as fast and slower growing broiler chickens, broiler breeders, laying hen and turkeys. Oechsle *et al*. (2016) reported about the use of chicken skin collagen for co-extruded sausage casings. Their conclusion was that the chicken skin could be a suitable collagen source for collagen dispersions. As indicated before, a commercial poultry collagen dispersion is currently available on the market, but at a very low volume (Anonymous, 2021). In any case, there is no scientific data indicating whether or not other commercially available skins of

different poultry species are suitable to serve as a collagen source for co-extruded sausage casings. Collagen obtained from various poultry species may feature different gel properties and thus yielding casing/films with different functionalities, such as flexibility and strength. Furthermore, different gel properties could also lead to differences in performance, e.g., extrudability, in the co-extrusion process. The use of chicken skins as a collagen source in the co-extrusion application is a very interesting one, especially for sausage manufacturers producing for the halal/kosher market. They are currently using natural sheep casings as material and could replace this for less expensive chicken collagen dispersion. Providing them with information about the performance of the gel could enhance their motivation to move from a batch to a continuous sausage production process.

7.3 Age

The animal's age influences the collagen structure. With increasing age, the degree of covalent crosslinks increases, due to lysyl oxidase-initiated crosslinking (Oechsle, 2016; Noorzai, 2020). This means that the soluble "young" collagen progressively changes into "mature" collagen. The alterations of the physical and chemical properties of collagen fibers, due to aging, are very distinct. The fibers become increasingly insoluble with age and their ability to swell in an acid solution decreases due to more crosslinks, which results in increased mechanical strength and stiffness. However, there is probably an optimum in the animal's age as the stiffness of the collagen fibers continues to increase, i.e., which is related to the level of crosslinking. This would eventually result in fibers with high brittleness and therefore lower tensile strength (Varnali, 2002). Currently, the optimum in the animal's age per species for obtaining good collagen quality of co-extrusion application is unknown. It is also unknown whether or not it is possible to alter this optimum by, for example, adjusting the collagen extraction process (e.g., variation in dwell times in NaOH solution) and thereby retaining the collagen quality. The animals' age becomes important when comparing collagen properties of bovine skin and chicken skin. Beef cattle is being processed at 18 – 36 months of age (Miller *et al*., 1983; Burke, 1980), whereas broilers are processed at 5-6 weeks of age (Jacobs-Reitsma *et al*., 1995). The collagen from beef skin has more crosslinks than broiler skin collagen and this may considerably affect the applicability of the broilers' collagen for casing production. Broiler skin collagen is easily solubilized during the collagen extraction process in the alkaline environment because the crosslinks are not very stable (Noorzai, 2020), resulting in different quality properties (e.g., lower mechanical strength) compared to collagen from beef cattle skin. No scientific data is available whether or not the skins of older poultry species (e.g., broiler breeder or laying hens, which are approximately 50-60 and 80-100 weeks old, respectively) would yield collagen quality characteristics suitable for the co-extrusion application. Beef skin collagen (currently used for commercial dispersions, prepared from beef cattle 18 – 36 months of age) is stable, due to

crosslinking, which makes it less prone to solubilization in high alkaline environment. Hereby, more fibrils and fibers are available to create mechanical strength in the final application, i.e., sausage casing. Miller *et al*. (1983) investigated the age-related changes in collagen of bovine corium and reported that increased biological age of different cattle breeds showed greater resistance to the action of acids, as measured by extractable collagen. They attributed this to the greater resistance to degradation in collagen of older bovines due to increased number of crosslinks. However, no scientific data is available how the collagen quality for co-extrusion purposes is affected when using collagen from older or younger cattle skins.

7.4 Collagen origin

The variability of skin tissue, used for dispersion production, is also an important factor, especially when working with cattle skins. The major parts of the skin are defined as "butt", "belly" and "neck" region. Skin parts located closer to the spine of the animal (butt region) are tougher (stronger fiber bundles) than parts located closer to the abdomen (belly region/that has loose fiber bundles). The neck part is the thickest part with a relatively open structure and with loose fiber bundles (Covington, 2009). The differences in collagen structure probably originates from the environment the animals were raised. The skin of animals, that spend most of the year outside, are likely to have a higher degree of crosslinking due to sunlight exposure compared to animals that have been raised indoors. For example, steers in Latin America have a higher degree of skin crosslinking versus steers in Scotland (Norwood, 2021). The number of hours of sunshine that the skin of these animals receive is greater than those from Europe. So, depending on which parts of the skin collagen is selected to make a dispersion, different properties can be found. With respect to sustainability and the reuse of residual material from animal processing operations, it is an important aspect of the dispersion preparation to try and make the non-uniform skin structure of skin trimmings as uniform as possible in the final collagen casing (Noorzai *et al.*, 2019; Matinong *et al.*, 2022). Noorzai *et al.* (2019) investigated how to maximize the collagen yield from various waste bovine skin sources, but did not look at the application of the collagen. So, it is unknown what a composite sample of all kinds of trimmings has on the collagen quality of the dispersion for co-extrusion. A too inhomogenous dispersion (e.g., different tissue structures with different swelling behavior) with respect to viscosity can lead to problems with extrusion of the dispersion. Next to animal skins, also other organs, like trimmings of intestines and urinary bladder could serve as a potential collagen source. However, no scientific data is available on the usage of these tissues in the co-extrusion casing industry. The urinary bladder is traditionally used in Southern-Europe as a casing for large diameter sausages, but not as a collagen source for dispersion production in the co-extrusion process. The application of both materials gives the opportunity to upgrade residual materials from animal processing operations.

8. Aim and outline of the thesis

Due to rising cost and reduced availability of sheep and pork intestines, as well as the increased need for kosher and halal products and the request for consistent sausage quality characteristics, collagen producers are searching for alternative collagen sources and factors influencing the quality of the collagen to be used for casing production (Zajac *et al.*, 2021). The factors that might play a role in the collagen quality of livestock to produce collagen dispersions for co-extrusion application is currently mostly unknown. No scientific data is available on the role of age and breed of different species and of different origins of collagen (skin, bladder, intestines) on the collagen dispersion quality suitable for the sausage co-extrusion process. This information is of great importance to both machine manufacturers and sausage producers and can help design new processes and equipment or optimize existing ones. Moreover, sausage producers can use the information to make it easier to choose which type of collagen dispersion is suitable for each specific product. In addition, the valuation of by-products from animal processing operations is an important aspect to improve sustainable animal production. Today, potential materials from alternative animal species are considered as waste or are being processed by the pet food industry. However, these materials could potentially be suitable for casing manufactures.

The overall objective of this thesis is to provide fundamental knowledge for understanding effects of biological factors on collagen quality of livestock animals for producing co-extruded casings. This includes three aspects: 1) investigating suitability of different animal species (poultry, cattle, pork); 2) investigating suitability of different collagen origins (skin, intestines, bladder); 3) investigating suitability of different breed/strain of animals. To obtain this knowledge, fundamental differences in collagen matrix structure will be investigated in relation to properties required in the co-extrusion process, as collagen is the main source for producing edible casings for the food industry.

Main question:
How do biological (animal-related) factors of livestock influence the quality of the collagen matrix?

The structure and the outline of the thesis is shown in Figure 6, using a matrix structure.

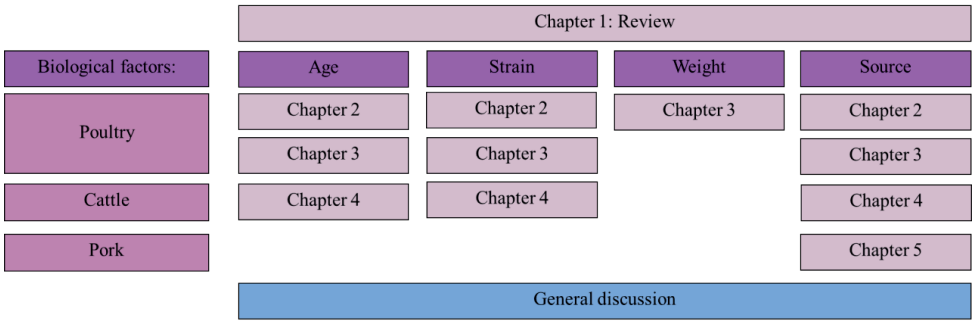


Figure 6. Structure and outline of the thesis.

Chapter 1 presents a literature review, focusing on collagen use to produce co-extrusion sausage casings. The review starts with some background information about the history of sausage production, followed by the description of the different casing types. Subsequently, the co-extruded collagen casing is discussed in more detail, focusing on the collagen extraction process from bovine skins (most used source today). Next, the different steps and the advantages of the co-extrusion process are discussed. Furthermore, various aspects that influence collagen properties and other potential collagen sources for sausage casings are discussed.

Chapter 2 evaluates the properties of different poultry skin sources for their potential usage in the co-extrusion process of sausages. Chicken breast skin from different aged poultry species: fast-growing broiler chickens (42 days old), slow growing broiler chickens (56 days old), broiler breeders (52 weeks old) and laying hens (100 weeks old) are harvested and collagen dispersions are prepared. Since each chicken type is slaughtered at a different age, poultry type is confounded with age.

Chapter 3 presents effects of broiler weight and strain on skin collagen characteristics and their applicability for co-extrusion sausage casings. The experiment had a 2x2 factorial design with two broiler chicken strains (fast growing and slow growing) and two different body weights (1.6 and 2.2 kg).

Chapter 4 evaluates different cattle skin sources for producing co-extruded sausage casings. Cattle skins from different types (American Calf, Dutch Heavy Veal, Danish Ox or Heifers and Heavy German Cow) are used to produce collagen dispersions. Since each cattle type is slaughtered at a different age, cattle type is confounded with age.

Collagen dispersions described in Chapters 2, 3 and 4 are evaluated for their biochemical, rheological, and mechanical properties, and the results are related to the co-extrusion process.

Chapter 5 evaluates collagen dispersions prepared from porcine urinary bladder and intestine as raw material for co-extruded sausage casings. The results described are collected over consecutive experiments as each experiment consisted of another developmental step. This Chapter reveals data that together with the secondary cooking properties (i.e., of sausages stuffed into these casings) reflects the potential use of these raw materials as sources for co-extruded sausage casings.

The **General discussion** summarizes the main findings and conclusions from all experiments. It discusses how and which the included biological factors (species, breed/strain, age, and collagen source) influence collagen quality to produce co-extruded sausage casings. The general discussion concludes with a summary of the main findings and opportunities for further research.

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CHAPTER 1

Collagen use for co-extruded sausage casings – A review

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Abstract

Background: In today's sausage production, various types of casings are employed including: natural, manufactured collagen, cellulose and plastic, as well as the new type of co-extruded casings made of collagen, alginate or alginate-collagen hybrids. Casings play important functional roles in sausage production from stuffing right up until the consumer eats the product. The selection of the right casing is critical as it influences the integrity, size and shape of the sausage while converting the soft, flowable raw meat batter into the desired semi-rigid sausage. The two most important casings' physical properties are their barrier properties and mechanical strength; both strongly affect consumer's perception of bite/ snap and flavor.

Scope and approach: Currently edible casings include natural sheep and pig intestines as well as collagen originating from bovine skins. This review focuses on the various aspects of latter collagen used for co-extrusion production, of high-quality sausages in an economical way.

Key finding and conclusions: Currently these relatively new co-extrusion gels come only with basic information about pH, protein content, and microbial counts, but nothing about physical characteristics such as viscosity, work to extrude and shear thinning properties. This is important as variations in collagen structure and functionality can be the results of environmental factors such as nutrition, housing, as well as age and genetics. Overall, the rising costs and shortage of intestines and increasing need for Kosher and Halal products, is putting pressure on collagen manufacturers to look for alternative sources with best performance, as described in the review.

1. Introduction

1.1 History of Sausage Making

Sausages are believed to be one of the oldest form of meat processing. While it is unknown when exactly the first sausage was made, there is evidence they existed for at least several thousand years. The origin of sausage making probably began when people learned that salt is an effective preservative. Sausage making evolved as an effort to economize and preserve meat that could not be consumed fresh at slaughter (Marchello *et al.*, 2017). A record of a cooked meat product stuffed into a goat stomach was found in the ancient city of Babylon (Anonymous, 1990). There is also mention of sausage in the oldest Greek cookbook which dates back to 228 BC (Tauber, 1982). In ancient Rome, 'farcimina' (sausages) were made and flavored with oriental spices, such as, pepper, cinnamon, ginger, and nutmeg (Jensen, 1953). By the late medieval period, the demand for sausages had outgrown the supply of casings, and salted casings were priced as delicacies in Europe (Fröhner, 1921). The Industrial Revolution led to advancements in new sausage production processes, but the custom of using natural casings did not change (Schutz 1921a; 1921b; Harper, 2013).

In the early 1900's, after the publication of the book named 'The Jungle', the United States introduced tougher inspection laws for the meat industry as well as the 'national pure-food law' (later named 'The Food and Drug Act'), which marked the beginning of the modern era of the American meat industry. Even prior to that, the 1871 German regulations, forced many German cities to construct public slaughterhouses, which helped Germans to reach high hygienic and technological standards. This was also a start for European cities to centralize animal slaughtering in their municipalities. This enabled an efficient utilization of various by products, including the processing of intestines into sausage casings (Savic & Savic, 2016).

As the meat packing industry continued to evolve, sausages became increasingly popular and hog casings became an important commodity. This led to the start of the modern-day casing suppliers. Until the 1960's, animal and cellulose casings were the only type of casing used. However, the 1960's saw the introduction of the first artificial sausage casings made from regenerated collagen. The fusion of traditional sausage making art together with advances in man-made casing manufacturing started what could be called the modern Sausage Age; i.e., a period in which sausages stuffed into attractive and functional man-made or natural casings became popular all around the world. This was the time when sausages reached great international popularity, with various regions developing their own unique sausage in natural or man-made casings (Savic & Savic, 2016).

Today, many types of sausage casings are used, including natural, manufactured collagen, cellulose, and plastic as well as co-extruded casings made of collagen, alginate or alginate-collagen hybrid casings (Harper, 2013).

1.2 Types of Sausage Casings

1.2.1 Natural casings

Sausages have always had an important place in the human diet due to their long shelf life (e.g., dry sausage), unique texture, flavor, nutritional density, and price. Sausages can be relatively inexpensive processed meat products resulting in many different varieties based on preference, culture, and climate.

Traditionally, sausage meat has been stuffed into intestines derived from pigs, sheep, and cattle (Wijnker, 2009). Sheep casings are obtained from the small intestine; note: the large intestines are not used for casings. The collagen content in the intestines of young sheep is higher than in the same intestinal section of a mature animal, however the intestines of the mature animal is always tougher. This is due to a higher proportion of stable crosslinks within collagen fibers of a mature animal (Savic and Savic, 2002). Sheep runners are fermented prior to the cleaning process, in contrary to hog casings. After the manure is removed/ stripped from the intestines, the runners are placed in cold storage overnight to allow the mucosa to degrade for easier removal, while the submucosa becomes more hydrated; the latter permits manual or machine sliming without damage. Sheep casings are not reversed (turned inside out) during processing, while beef casings are (Wijnker, 2009; Savic and Savic, 2002). Runners are never stored more than 2 days because prolonged storage will weaken the resulting casings. By no means should the casings be frozen during storage as they would lose their elasticity and firmness (Savic and Savic, 2002). Koolmees *et al.* (2004) described that after storage the runners (about 25 to 27 m long) were placed in water at 15 to 20°C to facilitate the removal of the mucosa. Later the cleaned casings are stored in salt. Sheep casings, as well as other natural casings are soaked in water prior to stuffing the sausage mix. This treatment removes part of the salt and the casing wall becomes more elastic, as the collagen fibers absorb water (Heinz and Hautzinger, 2007). Cleaned casings containing only submucosa, support stresses in all directions, which allows them to adapt to the extension and retraction of the sausage mass during processing and storage.

In the case of pig intestines several parts can be processed into casings; e.g., stomach, bungs, small and large intestines. The most important are the small intestines. The cleaning and preservation are similar to the procedure employed for sheep casings. Small runners are first pressed successive between rollers, which removes epithelial layer and usually both basement membrane and muscularis mucosae, as well as the muscle

layers and the serosa; i.e. leaving only the collagen rich submucosa. The submucosa of pig casing contains a network of collagen and elastic fibers, blood vessels and is less brittle than beef casing, which contain both the muscularis and mucosa layers. During cleaning of the intestines, the slime can be harvested for the pharmaceutical industry and used for the manufacturing medicines like heparin (anti-coagulant). Small diameter casings, free of holes, are of "A" quality and are used for fine emulsion type sausages. The "B" quality casings should be of good strength and of other physical properties, however they may have some small holes. They are used for coarse ground products such as fresh pork sausages (Escoubas *et al.* 2010; Smits and Keizer, 2003; Heinz and Hautzinger, 2007; Savic and Savic, 2002).

Beef casings are also used for sausage production. However, due to several cases of Bovine Spongiform Encephalopathy (BSE), European casings have been designated as specified risk material and therefore banned from human consumption completely in 1997. Overall, BSE infectivity has so far only been confirmed in the distal ileum and not in any part of the intestinal tract used to produce beef casings. Although BSE has not been identified in any other part of the intestinal tract, the production of beef casings in Europe was terminated and currently only imports from countries with a negligible BSE risk are allowed. While sheep and hog casings are typically eaten with the sausage, beef casings are usually removed prior to consumption as they are thicker and tougher/ hard to chew (Wijnker, 2009; Wijnker, Tersteeg, Berends, Vernooij, & Koolmees, 2008).

After cleaning, the casings are also stored in salt solution to lower the water activity, which inhibits microbial growth and preserves the casing (Savic and Savic, 2002; Ockerman, 1988; Smits and Keizer, 2003; Heinz and Hautzinger, 2007).

Natural casings are perceived to have the highest quality due to special characteristics; such as bite, knack, curvature, snap. Currently, not all these characteristics (e.g., permeability, pressure resistance, smoke absorption, and flexibility) can be reproduced by artificial casings. Because of that, natural casings are often considered as the gold standard for sausage casings and are expected to remain the preferred casing for traditional and some premium sausages (Anonymous, 2018; Savic and Savic, 2016).

1.2.2 Manufactured collagen casings

The limited supply of animal casings and their relatively high price encouraged the development of alternative type of casings (Kramlich, Pearson & Tauber, 1973). Development of manufactured collagen casings began in 1925 and production started in 1930. In 1960, more edible collagen casing came into the market (Savic & Savic, 2016).

Manufactured collagen casings are composed of both fibrous and solubilized material that is extracted from hides, bones and connective tissue (Ratanavaraporn *et al.*, 2008). The production of these casings is a proprietary process (Barbut, 2010). However, several steps are typically used across the board. In the case of beef collagen casing, the first step involves separating the corium layer from the rest of the hide, followed by decalcification and grinding. Acid is then added to induce swelling of the collagen and the so called collagen dough is extruded using either a 'wet' or 'dry' extrusion process (Savic & Savic, 2016). The 'wet' extrusion ('American' process) typically uses H_2SO_4 while the 'dry' process ('German' process) typically uses HCl (Savic and Savic, 2016). Crosslinking agents, such as glutaraldehyde or other aldehydes (e.g., from liquid smoke) may be added to control the extent of crosslinking and improve the strength of the casing. Cellulose fibers can also be added to improve the casing's strength while plasticizing agents, such as glycerol and sorbitol, are often added to reduce the casing's brittleness (Savic & Savic, 2016; Ustunol, 2009). Overall, the latter molecules work by decreasing the intermolecular forces along the polymer chains allowing for more chain mobility and flexibility.

Manufactured collagen casings have gained popularity because they possess many similarities to traditional animal casings, including the fact that they are edible. The cooking characteristics and tenderness of sausages stuffed in manufactured collagen casings are quite like those in natural casings (Savic & Savic, 2016). In addition, they show improved uniformity, strength, flexibility, hygiene and are easy to use during filling, portioning and slicing (Kutas, 1987, Osburn, 2000; Savic & Savic, 2016). Manufactured collagen casings do not have size variations or weak spots like natural casings. Overall, they are easier to work with compared to natural casings as they are very uniform (important for portion control), allow using higher filling pressure within fast stuffing machines, less expensive to buy, and require less labor (i.e., arrive as a tube that can be put directly onto the stuffing horn) (Savic & Savic, 2016). Manufactured collagen casings also result in a cleaner, more sanitary product than natural casings (i.e., no/ minimal microbial load due to exposure to the high pH during manufacturing). They require no soaking prior to stuffing and can be stored longer than natural casings (Savic & Savic, 2016). However, both manufactured collagen, natural casings are of a finite length, and thus sausage stuffing remains a batch operation (Harper, 2013).

1.2.3 Cellulose casings

In 1870, German army sausage suppliers first used parchment paper casings for their sausages. These casings were the first known type of artificial casing used. Although they are rarely used today (except for some ethnic specialty sausages), other types of cellulose casings are common. Manufactured cellulose casing are very popular for high volume products such as frankfurter type sausages, bologna and salami. These casings

are very strong and can be used on highly automated equipment. Because of their uniformity and the ability to control the degree of stretching, portion control is easy. Cellulose casings are non-digestible and must be peeled off prior to consumption. In the case of small diameter products (hot dogs), the casing is peeled off at the plant by an automated high-speed peeler. In case of larger diameter products, like salami, the casing is usually peeled off by the consumer. Some cellulose type casings are coated with a protein layer to improve the adherence to the product. This is especially important in case shrinkage of the meat batter is expected (e.g., semi/ fully dried sausages). Cellulose casings are water and smoke permeable unless they are coated with plastic (Barbut, 2015). Cellulose casings can be divided into three groups; non-fibrous cellulose casings, fibrous cellulose casings, and plastic (e.g., Polyvinylidene Chloride; PVDC)-coated fibrous cellulose casings (Savic & Savic, 2016). Small diameter non-fibrous cellulose casings are designed to give maximum uniformity in diameter and are mainly used to produce skinless wieners, hot dogs and smoked sausages (casings removed at the plant after heat processing). Fibrous casings consist of a cellulose hydrate (produced from a cellulose xanthate derivative) that has been reinforced with regenerated cellulose fibers (Savic & Savic, 2016). Fibrous casings come in three basic types; regular, easy peel, and moisture proof (PVDC coated). Easy peel and PVDC coated fibrous casings are used for cooked cold cut sausages, as well as other large diameter products (Thode, 2011; Harper, 2013).

1.2.4 Synthetic polymer casings

Today, sausage casings are also made from synthetic polymers; such as PVDC, polyester, polyethylene, polyamide, polypropylene or combinations of these materials (Thode, 2011). Synthetic casings are a good choice for large-diameter sausages since they are known for their relative strength and uniformity. Another advantage of plastic films is that they offer protection against oxidation since they are impermeable to oxygen and this can significantly help to enhance products' shelf life. However, the properties of these casings vary depending on the type of polymer, thickness, additives used, as well as post-processing treatments. Like cellulose casings, synthetic polymer casings are indigestible and must be removed prior to consumption. Typically, these casings cannot be smoked, although recently smokeable synthetic casings, with small holes, have been developed (Barbut, 2015; Savic & Savic, 2016).

1.2.5 Co-extruded collagen casing

Co-extrusion is a production method, which avoids the intermediate stages of preparing and storing pre-made casings. Unlike traditional sausage production, where casing is stuffed with meat, co-extrusion introduces liquid/ semi liquid material (to the sausage surface) that is later gelled in place. Initial reports and patents regarding the technology started to appear about 40 years ago. However due to various technical challenges, co-extrusion has only started to be used, on a large scale, about 10 – 15 years ago. It

was in 1982, that Unilever Ltd. London-Rotterdam developed co-extrusion technology, which allowed forming the casings directly on the sausage surface. However, problems with casing stability as well as the high investment cost and complexity of the process deterred sausage manufacturers from implementing the technology (Savic & Savic, 2016). During the late 1990's Townsend Engineering significantly improved the co-extrusion process and within the last 15 years several major North American and European companies adopted the technology on a large scale (Savic & Savic, 2016). Today, co-extruded casings can be made of collagen, alginate, or an alginate-collagen hybrid material. Additives, such as, cellulose or potato starch, may also be added to the casing to improve certain functionalities.

2. Co-extruded collagen casings

2.1 Main source of collagen and structure

For (co-extruded) collagen casings bovine skin is the main source of collagen and why this technology will be reviewed in more detail below. Collagen is a by-product of the meat packing industry. It is obtained from skin of slaughtered beef cattle and makes an intermediate stop at the leather tannery, where the hair and dermal layer are separated from the collagen-rich corium layer. Wide varieties of skins go to tanneries for further processing and most are graded for leather use. The dermal or the grain section receives separate chemical and tanning treatments, which permanently stabilizes it and results in leather. The corium continues for conversion into either second-grade leather or edible products, such as collagen casings, food grade gelatin or pet snacks (Covington, 2009a).

The dermis/ corium is the layer under the epidermis, providing physical strength and flexibility to skin. The dermis/ corium is divided into two regions: the papillary dermis, composed of loose connective tissue containing hair follicles and glands. The reticular dermis containing large bundles of collagen interwoven in many directions. In general, 100 kg of skin contains about 40 kg of collagen and 60 kg of water (Covington, 2009a). The dermis/ corium thickness varies in different anatomical regions of the animal (Falanga *et al.* 2014; Savic and Savic, 2002). Covington (2009a) described the skin as anisotropic, i.e., its structure and properties vary over the surface. The parts of the skin can be defined in terms of the "butt", the "belly" and the "neck". The butt is defined as the region up to halfway from the backbone to the belly edge, and two-thirds of the way from the root of the tail to the neck edge. Within this region, the fibre structure is relatively consistent and hence the physical properties of the skin are consistent. The remaining regions to the side of the butts are called the bellies and the region beyond

the butt, towards the head, is the neck. The butt has a tight fibre structure, making the skin relatively firm and stiff. It is thick compared to the belly, but thinner compared to the neck. For gel production, only the bellies or the belly and the butt of beef cattle are used in one piece (Kempers, 2019). The bellies of all skins are the thinnest parts, with an open structure, making them relatively weak. The neck is the thickest part, but also has relatively more open structure. Due to the differences in collagen fibre structure, it is a challenge to make a constant quality collagen gel (Covington, 2009a).

Collagens can be divided into two major groups: fibrillar and non-fibrillar (Oechsle, 2016; Maxwell, 2007). This review focuses on the fibril forming collagens, as they are predominantly found in the skin, intestine, tendon and bone. A fibre is composed of tightly packed fibrils. Collagen types, which can form fibrils are types I, II, III, V and XI. The most abundant fibrillary collagen is type I, present in bovine skin and commonly used for making collagen casings (Oechsle, 2016).

The basic structural unit of collagen is composed of three polypeptide chains, also referred to as tropocollagen. Tropocollagen is the basic molecular unit of collagen with a general molecular weight of about 300 kDa, length of 280 nm and width of 1.5 nm (Brodsky, 2003). The polypeptides could have the same sequence, hence forming homotrimer molecules, or have different sequences forming heterotrimer molecules, depending on the collagen type (Oechsle, 2016; Maxwell, 2007). In collagen type I, the polypeptides are arranged in the form of a triple helix with two identical chains (α1) and the third, which differs to some extent in its amino acid composition (α2). The triple helix structure is stabilized by hydrogen bonds. All collagen types contain the sequence $(\text{glycine-X-Y})_n$ giving rise to α-chains. The X and Y can be any amino acid, but the positions are frequently occupied by proline and hydroxyproline, respectively. Hydroxyproline is in fact not a naturally occurring free amino acid but is formed from proline. The chemical change takes place after the protein is synthesized. Hydroxyproline is formed by an enzymatic post-translational modification of proline by the enzyme 4-prolyl hydroxylase. The hydroxyproline and proline provide rigidity in the triple helix structure and force the peptide chain into a left-handed helix. The smallest amino acid glycine allows tight packing of the three α-chains and provides flexibility to the peptide backbone (Savic *et al.* 2002; Chambers, 2004; Oechsle, 2016).

Areas within the collagen fibres, characterized by a lack of hydroxyproline and proline and subsequently a lack of triple helix structure are referred to as the telopeptide regions (at both the N- and C-terminal ends). The telopeptide region is rich in lysine and hydroxylysine residues and has three main functions; 1) stabilization of the molecule via intra-molecular and inter-molecular crosslinking, 2) crosslinking with other extracellular matrix proteins, and 3) formation of "sticky ends" by disulphide bonds to

aid self-assembly. Tropocollagen molecules overlap approximately one-fourth with its neighbouring molecule (67 nm) due to assembling in a quarter-staggered fibril array (Savic *et al.* 2002; Chambers, 2004). This characteristic banding pattern has a periodicity of 65-70 nm and is also known as the D-period (Hoogenkamp, 2015; Versteegden, 2017). Figure 1 shows the construction of a collagen fibre starting with the structural formula of glycine, proline and hydroxyproline. Followed by collagen triple helix forming micro-fibrils, which in turn assemble to collagen fibrils and fibres (Oechsle, 2016).

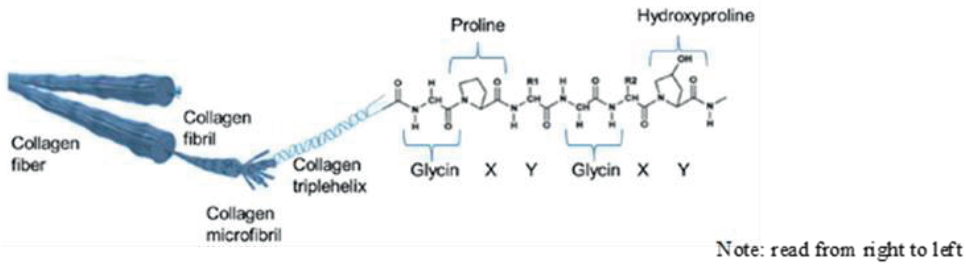

Figure 1. Systematic illustration of collagen triple helices forming micro fibrils, which in turn assemble to collagen fibrils and fibres (From: Oechsle, 2016).

2.2. Production of collagen from bovine skin

2.2.1 Dehairing process at the tanneries

The dehairing process of hides is carried out in tanneries and is discussed here as the chemicals used also affect the collagen later harvested from bovine skins. The aim of the dehairing and liming processes is to remove the hair, epidermis and to some degree the inter-fibrillary proteins, and thus to prepare the hide for the removal of loose flesh and fat, by the fleshing process. The most frequently used dehairing technique is based upon alkaline treatment of the hides. There are two version of the process, namely hair dissolving (burning) and hair saving (Covington, 2009a; Savic and Savic, 2002). In the hair dissolving process, a strong reducing agent such as 2% Na_2S is immediately added to the treatment vessel. The sodium sulphide rapidly dissolves in water to yield caustic soda and sodium sulfhydrate (NaHS): $Na_2S + H_2O$ -> NaOH + NaHS. This results in a direct attack on the disulphide bonds, which breaks down the keratin molecules in the hair. Due to the strong alkaline conditions, the disulphide bonds of the keratin molecule are quickly hydrolyzed and the hair is dissolved, beginning at the tip and then proceeding to the follicle. Softened hair stubble remains temporarily attached but the stubble is subsequently being removed as the soft follicular keratins are destroyed during the lime application that follows the sulphide addition. It is a rather simple technology with minimum process control required (Hood, 1987; Covington, 2009a).

The best-known method for hair saving in the leather industry has been described (Covington, 2009a; Hood, 1987) as follows: The skin is impregnated with sodium hydrosulphide for 2 h, to drop the pH from 11.2 to 8.5. After 2 h, the solution is drained and calcium hypochlorite is added for 5 min. Then lime is added to raise the pH to 12.5. A rubbing action on the skin is necessary to remove the hair, which can be recovered as a valuable by-product. A re-lime step, with sulphide, is carried out to dissolve residual hair. It is a more complicated technology than hair dissolving, which requires a more precise, process control especially pH control.

2.2.2 Liming and its effect on collagen structure

Liming results in swelling of the skin due to the high pH. The skin can be swollen by three mechanisms:

- Charge effects, based on breaking salt links and creating more charges within the protein structure
- Osmotic swelling, caused by the imbalance between the ionic concentration outside and inside the skin
- Lyotropic swelling, caused by disruption to the collagen structure by molecules such as lithium bromide, that can be inserted among the hydrogen bonding

In the case of alkali swelling, the mechanisms are charge effects and lyotropy. The net effect of alkali on collagen is breaking the natural salt links and making the protein anionic. The repelling effect of the anionic centres causes the collagen structure to open up, allowing water in, which is observed as swelling. The opening of the collagen structure occurs in response to the deamidation by hydrolysis of the side-chain glutamine and the asparagine residues. This results in a sharp decrease of the isoelectric point (pI) of the native collagen fibers (Hood, 1987; Covington, 2009b; Lischuk et al, 2006, Maxwell et al, 2006). Long alkaline treatment yields collagen with pI values closer to 5.0, whereas mildly treated collagen records a value of 7.8, and acid treated collagen a value of 9.0 – 9.2 (Hood,1987). It seems that the prolonged lime treatment, at pH above 11, results in fully anionic structures. In such a case, the fibrils will absorb water, swell and open up. At the end, the fibers never regain their native associations even though collagen is later passed through a series of different pH and solvent changes. The collagen suitable for producing casings no longer possesses the native molecular properties after the hair removal process. The structure is permanently changed, and the fibers have an increased contact area that can later be used for cross-link formation. Overall, cross-linking is the chemical basis for the permanent stabilization of collagen casing after drying (Hood, 1987; Savic and Savic, 2002; Varnali, 2002; Maxwell et al, 2006).

The consequences of lime treatment are:

- Separation of collagen from its associations with non-collagenous proteins and carbohydrates
- Altering collagen ionic nature and solute-binding properties
- Destroying the native cross-linked and hydrogen bond structure, by the calcium ions.

This does not say that the lime treatment has a negative effect; in fact, there is a need for disruption of some of the native structures and biochemical associations to make an acceptable casing. Overall, this means that physicochemical data derived from isolated native collagen is of limited value when trying to interpret performance properties of collagen casings (Hood, 1987; Covington, 2009b) and that is one of the industry's main challenges today.

2.2.3 Swelling of collagen fibers due to liming

Figure 2 illustrates how the standard tannery lime treatment can affect the swelling of bovine hide collagen. The graph shows the percentage swelling against different pH values for purified collagen and for alkaline treated collagen (lime treatment). As can be seen, the water absorption properties are pH dependent and the liming process intensifies the swelling maxima experienced under alkaline and acid conditions. Of special interest for the manufacturers of collagen casings is the swelling behaviour at pH 3. At this particular pH the collagen shows maximal gel viscosity, at any given solids level and this encourages uniform fibre dispersion and flow properties of the collagen dough used to create the casings during the extrusion process. Besides the pH effect on viscosity, other physical changes also occur; e.g., collagen is more firm and translucent above pH 9 and below pH 4.7. In the middle pH range (pH 5 – 9) where swelling is minimal, the collagen exhibits white opaque colour, since water absorption is minimal in this region. Overall, liming effects may be enhanced by time, temperature and pH increase (Loders Croklaan, 2004; Hood, 1987; Varnali, 2002; Covington, 2009b).

2.2.4 Further processing of beef hides by collagen casing manufacturers

The liming process should be executed as long as necessary (few h to several days) to achieve the required characteristics for the final application. Prolonged treatment under high alkaline conditions eventually destroys the triple helical structure and fibrillar integrity, thereby yielding low molecular weight polypeptides, which are useless for collagen extrusion (Hood,1987). Properly delimed corium has a final pH of 3.5 – 5.0, which can be achieved by careful chemical treatment with $(NH_4)_2SO_4$, NH_4Cl and/ or acidic buffers (lactic acid). Deliming process is essential to achieve the desired balance between elasticity and strength of the collagen fibers (Hood, 1987). Lime treated corium may contain 0.3-1.5% Ca^{2+}, while properly delimed hide contains 0.2% or less. The deliming

process is ultimately dependent upon the swelling phenomena of collagen, which was previously explained. Calcium removal, within edible corium destined for extrusion, is essential, so that residual $Ca(OH)_2$ does not interfere with uniform swelling of the fibrils (Hood, 1987). After deliming, the hide is homogenized and deaerated, followed by the addition of acetic/ lactic acid and packaging of the gel (Loders Croklaan, 2004).

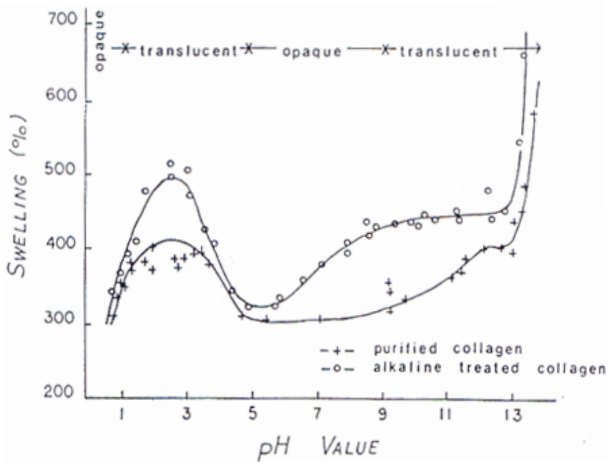

Figure 2. Effect of pH on extent of swelling in purified and alkali-treated bovine collagen (From: Hood, 1987)

2.3 Setting of pre-made and co-extruded collagen casing

The resulting collagen gel has to be formed into pre-made or co-extruded casing. The first is produced in specialized facilities where the gel is forced into an extrusion die which is creating the casing (at the desired diameter), initially "suspended" in a stream of warm air inside a closed environment/tunnel. The air is saturated with a cross-linking agent such as glutaraldehyde, which induces bonding among adjacent collagen molecules. The tubular casing then travels through a hot air zone to help dry it out. Later it is washed with water to remove excess of the cross-linking agents used (i.e., no glutaraldehyde can be incorporated into food). The casings can then be colored, and coated with a plasticizer, such as glycerol, to make them easier to handle at the meat processing plant. The casing is then shirred into sticks that can measure up to 20 meters per stick.

With co-extruded casings, the collagen gel (usually 3-6% protein) is sent to the meat plant where it is co-extruded on top of the sausage meat coming out of the extruder. In the co-extrusion process, a thin layer of casing material is extruded onto the meat batter as it is coming out of the extrusion nozzle. Figure 3 shows an overview of the extrusion technology, and the system setup is provided. The co-extrusion head is equipped

with two counter rotating cones (Figure 3). The resulting alignment depends on the gap between the two cones and their relative speeds. Figure 4 shows the resulting alignment with a setting of both cones rotating at 200 rpm. It demonstrates that the fibers deposited within the outer layer are positioned at about 40-degree angle to the product's long axis mostly. The middle layer shows random orientation, while the inner layer shows -30-degree orientation (i.e., because of its proximity to the inner rotating cone). Orienting the fibers improves the mechanical strength of casings by reducing the probability of casing splitting later during the cooking process (Ustunol, 2009; Hoogenkamp *et al*, 2015).

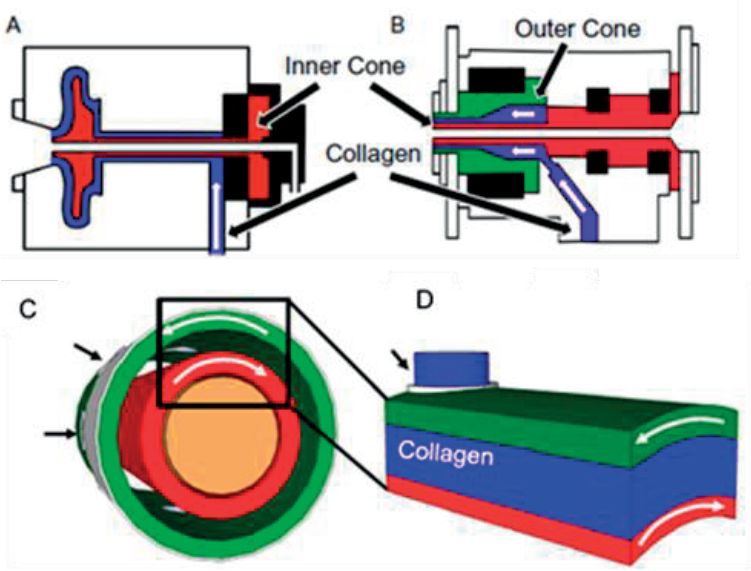

Figure 3. Overview of the extrusion technology and system setup. (A) Single cone extruder design and (B) counter-rotating cone extruder design, that can be used for collagen extrusion. Either one or two rotating cones can be used to influence the fiber direction. The inner cone (red) and the outer cone (green) are the rotating parts between which the collagen gel (blue color) is extruded. White arrows show the direction of collagen flow. (C and D) Cross-section of the extrusion head, with counter-rotating inner (red) and outer (green) cones molding the collagen film during extrusion (Hoogenkamp *et al.* 2015).

Producing co-extruded casings with hydrocolloid gums, such as alginate, requires a different extrusion system. As alginate is composed of two monosaccharides, guluronic acid (G) and mannuronic acid (M) and able to form a gel in the presence of polyvalent metal cations (Harper, 2013), there is no need to provide orientation during extrusion. Today hybrid casings made of, for example, collagen and alginate are also available, and they require the counter-rotating extrusion to align the collagen fibers. Other polysac-

charides that have been tried, as additives to collagen and/ or alginate include gums, such as carrageenan, which can provide some stability to the final casing (Bontjer *et al.*, 2011; Visser 2012; Ioi, 2013).

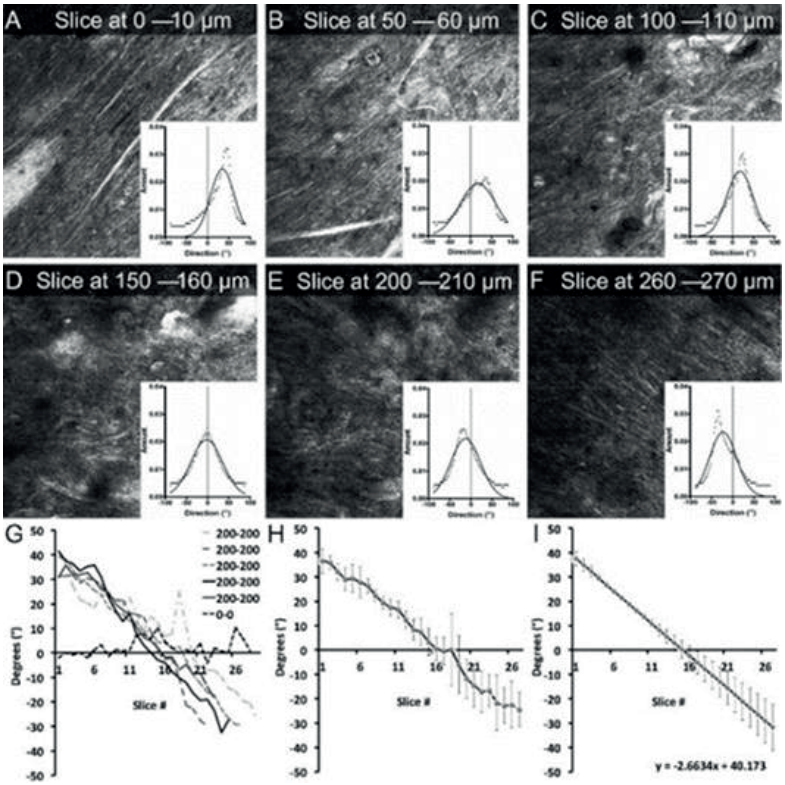

Figure 4. Directional organization of extrusion-generated 3-D collagen films. (A–F) Second harmonic signal images and quantification of fiber orientation (graphs, inset) from individual slices obtained at the indicated depth position of the image stack from films generated at 200–200 rpm cone speeds. Along the *x*-axis, the fiber orientation is shown (in degrees) where the collagen fiber orientation in the slices is measured and subsequently fitted with a Gaussian curve (solid lines). The peak of the curve represents the dominant fiber direction; (G) dominant fiber directions from speed 200–200 plotted against a direction for 0–0 control cone speed; (H) average dominant fiber direction profile of all 200–200 speeds combined. Error bars represent the standard deviation. The graph's solid black line changes to a dotted line from slice 21 to slice 27, which represents a deviating number of available data points (lower than $n = 5$); (I) the trendline average from combined 200–200 cone speeds. (Hoogenkamp *et al.*, 2015).

Once the casings have been extruded, subsequent treatments are required to stabilize them onto the product. In the case of collagen, the first step is de-watering, which is done by exposing the collagen coated product to a brine solution (e.g., 20 – 30% NaCl),

which removes some of the water from the casing (to get the collagen molecules closer together). The sausages can be either drenched or sprayed with saturated NaCl solution to dehydrate and harden the casing (Kobussen *et al.*, 2000; 2012). This also allows the collagen to conform to the shape of the meat batter. Further stabilization is performed by air-drying as well as cross-linking with smoke condensate and/ or other agents. Smoke is used because of the presence of aldehydes, which are capable to crosslink the proteins. In addition, the smoke adds flavor, colour and preservation (i.e., wood smoke has over 400 chemical compounds); (Toledo, 2007). As for sausages with alginate casings, the sausages are drenched in a calcium chloride solution (10 – 15%) to quickly form a casing. Hybrid alginate/ collagen casings are first gelled with calcium chloride solution followed by air-drying and crosslinking with liquid smoke. After the dehydration step the casings are of sufficient strength to undergo crimping or linking. Linking is accomplished by squeezing the sausage rope to define the individual sausages' length and weight. The crimper consists of an upper and lower v-formed guides. The crimping process involves the v-cutouts slowly closing on the sausage. The guides displace the meat within the coating until there is only a thin neck of casing, which can then be cut (Bradshaw and Taylor, 1971; Loi 2013).

Overall, there are a few different co-extrusion collagen gels/ dispersions on the market, with different size fibers and adjusted to different pH values by different acids. The pH values of the commercial samples range between 2.04 to 2.67. For example, gels can be prepared with HCl (note: this is at the last stage after washing off the alkaline solution; see previous text), while other gels can be prepared with lactic and acetic acid. These collagen gels also show different viscosities; measured as work required to extrude them through a 7mm opening die (3.19 – 5.16 Nm; Barbut and Ioi, 2019).

Today most co-extrusion gels come with very basic data about pH, percent protein, and microbial counts, but no data concerning physical properties such as viscosity, work to extrude, and shear thinning. Such data can help meat processors make more informed decisions, regarding a product and/or batch they would like to use. (Note: the batch issue is mentioned here because variations among batches can occur, due to the nature of this product, which is affected by animal age, breed, and preparation procedure. See next section for more details).

Overall, there is limited information published regarding co-extruded collagen casing's characteristics. Table 1 shows the mechanical properties of collagen films prepared by co-extrusion and compares cross-linking with smoke condensate (SC), and glutaraldehyde (GA). A smoke condensate (a 15 concentration) (Charsol Select 24P Liquid Smoke, Red Arrow Products, Manitowoc, WI, USA) dissolved in deionized water, and glutaraldehyde (EM Grade, Canemco, Canton de Gore, QC, CAN) solutions of 0.1, 0.5 and 1.0

vol.% GA in 1M HEPES buffer at pH 7.4 were used. As can be seen, there are quite some differences in the tensile strength and percent elongation among the commercial gels, regardless of the cross-linking agent used (Barbut and Ioi, 2019).

Table 1. Mechanical properties of cross-linked films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5) (Barbut and Ioi, 2019).

Collagen	Cross-linker[1]	Tensile Strength[2] (MPa)	Percent Elongation[2] (%)	Distance at Break[3] (mm)	Work to Break[3] (Nmm)	Thickness (mm)
C1	SC	0.67 ± 0.04^{a}	24.80 ± 1.23^{ab}	3.85 ± 0.22^{a}	2.75 ± 0.48^{a}	0.35 ± 0.01^{ab}
C2	SC	0.53 ± 0.02^{ab}	26.32 ± 1.18^{a}	3.39 ± 0.18^{a}	1.52 ± 0.14^{a}	0.30 ± 0.01^{b}
C3	SC	0.38 ± 0.05^{b}	22.41 ± 0.78^{abc}	3.21 ± 0.30^{a}	1.24 ± 0.34^{a}	0.34 ± 0.01^{ab}
C4	SC	0.32 ± 0.07^{b}	21.37 ± 0.99^{bc}	3.27 ± 0.35^{a}	1.23 ± 0.31^{a}	0.36 ± 0.02^{a}
C5	SC	0.39 ± 0.16^{b}	18.81 ± 2.08^{c}	2.59 ± 0.04^{a}	0.85 ± 0.10^{a}	0.38 ± 0.03^{a}
C1	GA	0.91 ± 0.17^{d}	26.26 ± 4.65^{d}	2.77 ± 0.52^{d}	1.79 ± 0.57^{d}	0.36 ± 0.03^{d}
C2	GA	0.66 ± 0.02^{e}	20.38 ± 1.43^{d}	2.34 ± 0.44^{d}	1.06 ± 0.37^{d}	0.45 ± 0.01^{d}
C3	GA	0.41 ± 0.13^{f}	18.95 ± 2.56^{d}	2.66 ± 0.39^{d}	1.35 ± 0.27^{d}	0.38 ± 0.05^{d}
C4	GA	0.61 ± 0.20^{ef}	24.18 ± 3.72^{d}	2.74 ± 0.29^{d}	1.52 ± 0.11^{d}	0.38 ± 0.04^{d}
C5	GA	0.60 ± 0.18^{ef}	22.04 ± 2.60^{d}	2.87 ± 0.60^{d}	1.91 ± 0.76^{d}	0.39 ± 0.05^{d}

[1]Smoke condensate (SC, Glutaraldehyde (GA). [2]Tensile test. [3]Puncture test.
[4]Means in columns with same letter are not significantly different p> 0.05: letters $^{a-c}$ refer to Smoke Condensate treated films; $^{d-e}$ Glutaraldehyde treated films.

It is also important to understand that the onset and denaturation temperatures of the raw collagen doughs after being treated with salt and the smoke condensate changes (Table 2). This is a clear indication that the collagen is significantly modified by the preparation process of the gel (see Sections 2.2.2 and 2.2.3 about liming and acidification). Overall, native collagen samples show a denaturation transition at about 60°C (Bernal and Stanley, 1986). After the liming, and acidification of the collagen, the denaturation point goes down to about 34°C (Table 2). Then the collagen goes through a second modification as it is exposed to the co-extrusion treatments (brining in a saturated salt solution and cross-linking with aldehydes). This results in the denaturation point basically doubling and reaching about 62°C (Table 2).

Also, acid type and pH influence final collagen structure and film functionality. Oechsle *et al* (2014) studied rheological properties of collagen suspended in phosphoric, sulfuric, hydrochloric and perchloric acid at pH 1, 2 and 3. The results showed that collagen entanglement increases with increasing pH values below the isoelectric point. This knowledge could be used to effectively modulate collagen structure and film functionality. For instance, highly entangled collagen matrices are more likely to be fabricated

into co-extruded collagen casings with high elasticity and tensile strength. These findings emphasize the importance of the collagen raw materials preparation steps for subsequent processing in either pre-made or co-extruded casings.

Table 2. Analysis of endothermic peaks from differential scanning calorimetry (DSC) thermograms. Five commercial collagen samples were tested as collagen dispersions and partially dehydrated/brined films: C1 (Collagen 1), C2 (Collagen 2), C3 (Collagen 3), C4 (Collagen 4) and C5 (Collagen 5) (Barbut *et al.*, 2020).

Collagen	Treatment	Onset Temperature (°C)	Temperature of denaturation (°C)	Enthalpy ΔH (J/g)
C1	Dispersion	33.54 ± 0.21	36.71 ± 0.51	5.33 ± 0.61
C2	Dispersion	34.59 ± 0.15	38.44 ± 0.06	3.05 ± 0.31
C3	Dispersion	34.26 ± 0.01	38.09 ± 0.08	4.12 ± 0.10
C4	Dispersion	35.41 ± 0.11	38.94 ± 0.02	3.93 ± 0.26
C5	Dispersion	33.45 ± 0.10	37.30 ± 0.21	4.45 ± 0.03
C1	Film	59.90 ± 0.23	64.87 ± 0.12	3.07 ± 0.55
C2	Film	58.40 ± 0.21	63.88 ± 0.57	1.76 ± 0.38
C3	Film	60.32 ± 1.61	65.00 ± 0.68	3.05 ± 0.21
C4	Film	58.22 ± 0.24	63.94 ± 0.61	3.06± 0.82
C5	Film	58.30 ± 0.40	65.34 ± 0.37	4.19 ± 0.37

Oechsle, et al (2015) found that collagen can be modified by adding chaotropic or kosmotropic salts of the reversed Hofmeister series. The study demonstrated that collagen entanglement and microstructure strongly depend on the ionic strength and type of salt. Whereby chaotropes form fine precipitates and kosmotropes leading to elastic three-dimensional networks. Higher salt concentrations increase the collagen–collagen interactions due to ions withdrawing the water from the collagen molecules. Therefore, salt addition is a convenient tool to modify collagen structure, rheology, and functionality for various applications. Nevertheless, modification of the collagen matrix by salt is a physical process, thus structures need to be later fixed by chemical reactions to preserve the changes in the conformation.

2.4 Advantages of co-extrusion process

The major advantage of the co-extrusion process is that it is a continuous operation as opposed to the traditional batch process, and thus capable of large production volumes (Smits, 1985). While the initial equipment costs can be high, the increased output and decreased labour costs are economically advantageous. The speed and uniformity of the co-extrusion process also make it appealing to meat processors (Bontjer *et al.*, 2011). With fewer people handling the product, there is a lower risk of microbial con-

tamination compared to traditional batch operations. Additionally, the amount of waste (i.e., casings ends; casings that break cannot be used for re-stuffing), and re-work are significantly reduced when using the co-extrusion principle (Anonymous, 2012). Since co-extruded casings are edible, they can easily be used for cook-in-bag processing. This prevents the risk of post-cooking microbial contamination (e.g., Listeria) from machines such as peelers, collators, and slicers (Harper, 2013; Barbut, 2015). Today, it is estimated that one third of the small diameter sausage production in the US is employing this technology.

2.5. Functional properties of co-extruded casings

Casings play important functional roles in sausage production from the moment of stuffing right up until the consumer eats the product. The selection of the right casing is critical as it not only influences the integrity, size and shape of the sausage but also assists in the conversion of raw flowable meat batter into the desired semi-solid sausage product (Savic & Savic, 2016). Casings are designed to accommodate a sausage manufacturer's quality and processing needs. The ideal collagen casing should provide sufficient strength to undergo processing, while remaining tender enough during consumption (Miller, 1983). It is not possible to create an all-purpose sausage casing suited for all types of sausages, as processing requirements can vary greatly. However, every sausage casing must fulfill some basic functional properties. The two most important are: barrier properties (i.e., permeability to water vapor, smoke, and gases) and mechanical strength. Several methods have been employed to objectively test the properties of casings (Barbut, 2010; Harper *et al.*, 2012, 2013; Hoogenkamp *et al.* 2015; Miller, 1983). Properties of interest also include caliber uniformity, light transparency, shrink ability, temperature resistance, color, peel ability, printability, texture, and potential use to carry functional ingredients (Harper, 2013; Ioi, 2013; Savic & Savic, 2016;).

Permeability depends on the casings type (e.g., composition, thickness), degree of extension, degree of water saturation, pH, and moisture content of the meat batter. Permeability describes the extent to which a permeating substance dissolves and then the rate at which it diffuses through the film. This migration of permeant is ultimately driven by concentration gradients. Polarity of a given material is obviously an important factor affecting permeability. In general, protein films have high permeability to polar substances such as water vapour, and low permeability to nonpolar substances such as oxygen, aroma compounds and oils (Krochta 2002). Collagen films, for example, have an excellent oxygen barrier at 0% relative humidity, but the oxygen permeability increases rapidly with increasing relative humidity (Lieberman and Gilbert, 1973). The migration of water and other substances into and out of the sausage is an important phenomenon, which produces complex effects on the texture, nutritive and marketing value of the sausage (Savic & Savic, 2016).

Variations in water content and the resulting water activity (Aw) strongly influence sensory quality and storage stability of the sausage. Changes in moisture content of the sausage are governed by the casing permeability properties. Water vapor permeability of the casing depends on aspects such as the level of dry and wet heat used during cooking, degree of casing extension, water saturation of the casing wall and pH of the meat batter. The casing permeability ultimately determines the weight loss from the product (Savic & Savic, 2016). In case of dry fermented products, a high degree of water permeability is preferred and co-extruded as well as manufactured collagen casings are often used.

With respect to shelf life, oxygen permeability of the casing is of crucial importance for the sausage. Oxygen in its ground state is relatively non-reactive, however it can be turned into reactive species, referred to as active oxygen forms or free radicals, which can accelerate lipid oxidation (Savic & Savic, 2016). Casing type and thickness will influence the rate of oxygen permeability, as well as the temperature and humidity. Microbial and biochemical spoilage can also be the result of oxygen, which is dissolved or entrained in the sausage mass. In addition, oxygen can cause protein oxidation, flavor oxidation, changes in color and loss of nutritional value. Consequently, reducing oxygen in packages (commonly done by vacuum packing) can help in retarding deteriorative reactions and extending shelf life of sausages. In a co-extrusion process, which uses "cook in the bag" technology, sausages are vacuum-packed, partially cooked (55°C), vacuum packed and then further cooked until core temperature of 74°C. The chance of any contamination is minimal and product shelf life is longer than for items produced using the more traditional cellulose casing method (Savic & Savic, 2016).

With respect to permeability, certain casings are readily permeable to smoke. Components in the smoke improve the flavor, can help peelability and extend the shelf life of the final product by lowering microbial growth (e.g., acid components in the smoke). Smoke components also produce a brownish/ golden color on the peripheral layer of the sausage; partially due to the Maillard reaction. The penetration of the smoke components is limited and ranges from 2 to 6 mm in depth depending on the intensity of the smoking process, application time, casing type, etc. It should be noted that the smoking process also depends on the humidity of the air and surface dryness of the sausage. If relative humidity is too high, the sausage will not be uniform in colour as some components will be washed off and result in an uneven colouring. If humidity is too low the surface will be unable to absorb enough smoke and the surface will have insufficient colour and lower flavor profile (Savic & Savic, 2016).

Next to permeability, the mechanical strength of the casing is a very important property. During processing the casings must be strong enough to hold the meat, but also be able

to expand (elastic properties) during cooking (Bakker *et al.* 1999; Simelane & Ustunol, 2005). The casing also helps control the gelling process of the meat batter inside, which is a prerequisite for structuring the sausage matrix. The tensile strength of a casing is defined as the maximum stress (force/ area) that a casing can withstand while being stretched before it breaks (Olivas & Barbosa-Cánovas, 2008). Tensile strength of a casing is determined by the collagen fibers, which are inter-molecularly, linked to each other via crosslinks. High tensile strength and elasticity are very important in certain type of sausages. These properties are also affected by factors, such as molecular weight, structure of the material, the number of plasticizer/ other additives and thickness of the casing (Savic & Savic, 2016). The presence of low level of cellulose fibers in manufactured collagen casing can make them stronger compared to casings made only from regenerated collagen fibers.

The mechanical strength of a casing is important as it influences consumer's perception of the bite/snap of the sausage (Bakker *et al.*, 1999). Harper *et al.* (2012) studied the texture and microstructure of four commercially available edible collagen casings and a natural sheep casing. Mechanical (textural) characteristics of the casings were evaluated by using shear, puncture and burst measurements. Shear force values were significantly higher for uncooked and cooked sausages prepared from natural sheep casing and one of the manufactured collagen casings tested, compared to those of the so called 'tender breakfast' and 'European wiener' manufactured collagen casings. Overall, it took more force to shear uncooked sausages width wise than length wise regardless of the type of casing. This phenomenon was not seen to the same extent in cooked sausages.

The elasticity of the casing represents the maximum force of casing extensibility. The elasticity of the casing (E modulus) can be measured with a texture analyser. The percentage elongation, at break, is calculated by dividing the change in length of the casings/ film, during stretching, by the initial length of the casings/ film, giving an indication of the elasticity (Wang *et al.*, 2007). Harper *et al.* (2012) also determined the elasticity, shear force and distance to break of these four commercially manufactured collagen casings and a natural sheep casing and reported that the distance to shear the casing of cooked sausages was lower than those for the uncooked sausages. A possible explanation is the fact that when the meat is cooked it gels, expands, and becomes a more solid like structure. This structure is exerting more force on the casing, thus limiting the ability of the casing to stretch, and resulting in a lower distance at break. Contradictory results by Amin and Ustunol (2007), reported that uncooked natural casings show a significantly lower percent elongation compared to uncooked manufactured collagen casings. However, the study by Harper *et al.* (2012) demonstrated that there is wide variety of manufactured casings on the market with different mechanical properties, and that can potentially explain the differences in the results between the two studies. The challenge

to match as many sensory properties of natural casings as possible remains a major priority of manufacturers of collagen casings (Savic & Savic, 2016).

2.6 Factors affecting collagen characteristics related to casing properties

2.6.1 Microbiology

Microorganisms can initiate collagenase activity, which could potentially destroy collagen fibre structure (Ponsen, 2010; Savic and Savic, 2016). Not all skins suitable for leather production are an appropriate source of edible collagen; e.g., in the US only skins originating from USDA inspected meat producing plants are acceptable; skins from animals with serious dermatological problems are ineligible.

2.6.2 Species

Every animal species (e.g., bovine, pig, chicken) has its own specific amino acid composition, which determines the final properties of the collagen. Hydroxyproline content is extremely important, because it affects the properties of any preparation made from collagen (Gomez-Guillen *et al.*, 2002). Animal species is important due to the differences in fibre density as well as weave pattern and therefore the type of possible application (Maxwell, 2007). Angele *et al.* (2004) evaluated the physico-chemical properties of equine- and bovine-collagen-based scaffold and found a highly significant effect of collagen type and crosslinking on degradation of the collagen samples by collagenase treatment. Crosslinked equine samples showed a significantly lower swelling ratio compared to bovine collagen samples. The amino acid composition of equine collagen revealed a higher amount of hydroxylysine and lysine. Shrinkage temperatures of non-crosslinked samples also showed a significant difference between equine (60°C) and bovine collagen (57°C). These properties are obviously of importance when producing collagen casings.

2.6.3 Age

Animal age is also important for casing production. Very young animals (fetal to 6 months) contain collagen with weak fibre strength, which is easily solubilised prior to extrusion, making it unsuitable for use in casings. Burke (1980) reported that heavy steers, aged approximately 2 years, produced acceptable material. Joseph (2003) stated that the average age for good beef collagen quality is 18 – 36 months. Age is also important when looking at collagen obtained from broilers. Collagen from young broilers is less cross-linked, as the degree of covalent crosslinking increases with advancing age; this in turn is related to lysyl oxidase-initiated cross-linking. As fast growing broilers are commonly processed at 5 to 6 weeks, these cross-links have not been fully developed yet (Oechsle, 2016). With increasing age, the formation of covalent bonds and crosslinks, between adjacent tropocollagen molecules, in the microfibrils and between adjacent microfibrils

increases. This means that the soluble "young" collagen progressively changes into "mature" collagen. Collagen and elastin undergo these continuous advancing molecular changes during life. The young collagen is capable of rapidly re-assembling to form a collagen triple helix. In contrast, mature collagen will only re-assemble over a period of days (Savic and Savic, 2016). The alterations of the physical and chemical properties of collagen fibers, due to aging, are very distinct. The fibers become increasingly insoluble with age, their ability to swell in acid solutions decreases due to cross-link formation, and both the mechanical strength and stiffness increases (Varnali, 2002).

2.6.4 Environment

Schwanz and Schnäckel (2007) and Harper *et al.* (2012) observed that animal casings can vary considerably in their quality. The difference in quality is possibly caused by the environment the animals are raised in. Schwanz and Schnäckel (2007) compared natural casings from Mongolia, Turkey, New Zealand, and Iran for their textural properties, such as tensile strength and elasticity. Differences in tear strength and elasticity were found and according to Savic and Savic (2016) they could be related to differences in climate. For example, New Zealand has a mild climate, where the animals have more fresh feed throughout the year and therefore sheep casings from that country are less strong compared to casing originating from Iranian animals (hot climate). Casings from Iran are stronger and according to Savic and Savic (2016) not suitable for all operations on sophisticated filling/ linking equipment.

The suitability of a collagen gel for a certain sausage product is determined by the degree of natural cross-links present in the collagen gel, which depends on the animal species, age and climate and is determined by the extent of the liming process/ number of cross-links broken down during the liming process (Ponsen, 2010).

2.7. Other collagen sources for co-extruded casings

The use of co-extruded sausage casings has increased due to the rising costs of natural intestinal-derived casings (Barbut, 2010). Moreover, hygienic production and high-volume sausage production requiring consistent quality characteristics, are becoming more important today. In addition, the increased demand for Kosher and Halal products puts pressure on non-pork collagen supply. Currently, beef collagen is the primary source for co-extruded casings. Chicken collagen could be used to supply the need (Cliche *et al.*, 2003, Munasinghe *et al.*, 2015; Oechsle *et al.*, 2016), since it is a common by product. While some chicken skin is incorporated into meat emulsion or used as a source of fat for soup preparation, not all is currently utilized by the industry; only some is used for animal feed. However, the large amount of collagen contained in chicken skin offers greater potential if diverted to casing production. Other potential chicken collagen sources are cartilages, bones and feet (Osburn, 2002). It should be noted that

currently chicken collagen is not used on a large scale for co-extrusion sausage production and is now mainly undergoing testing and improvement (Oechsle *et al.*, 2017). In any case, the reason that chicken collagen could be an alternative for bovine collagen is that they both contain the fibril-forming collagen types I and III.

Chicken collagen from commercial broilers is less crosslinked compared to bovine collagen. As indicated before, i.e., lower degree of covalent crosslinking as broilers chicken are processed at approximately 5 – 6 weeks, while the average age of beef cattle is 18 – 36 months. Oechsle *et al.* (2016) characterized chicken bone and skin collagen to determine their suitability for preparing sausage casings applying the co-extrusion technology. Chicken skin exhibited a fibrous microstructure with thin fibers and a lower ability to swell. Extraction of collagen from chicken bones was not successful, as large and firm fragments led to an inhomogeneous particulate suspension. Although the extraction did not succeed completely, the collagen fibers obtained from the bones were short. This observation was the reason for them to conclude that collagen from chicken bone is not suitable for making casings. Because of the short fibers, gel viscosity was found to be insufficient, mainly due to a low entanglement between the fibers. On the other hand, chicken skin collagen yielded more entangled long collagen fibers and a gel with higher viscosity, therefore providing the desired properties for casing extrusion and subsequent crosslinking. The researchers concluded that chicken skin collagen displayed the most suitable source of collagen for the co-extrusion process compared to the well-established bovine hide split collagen. The same was reported by Munasinghe *et al.* (2015) who indicated that opportunities exist for further development of chicken collagen film as an alternative to beef collagen. Overall, they investigated possible use of underutilizing chicken by-products, as an alternative collagen source, for industries such as pharmaceutical, cosmetics, biomedical materials and the food industry.

In a later study, Oechsle *et al.* (2017) reported on the possible modification of extruded chicken skin collagen films and telopeptide-poor collagen from bovine hide by the addition of NaCl (0.05 mol/kg) and/or partial substitution of collagen by 1.25% soy protein isolate. Salt addition to the initial gel allowed forming beef and chicken collagen films with high tensile strength and elasticity. In contrast, a substitution with soy proteins decreased gel and film strength. This weakening of the collagen networks could be compensated by adding NaCl, leading to more homogeneous gels yielding films with higher storage moduli upon extrusion. The compensating NaCl effect was more pronounced for chicken skin than for the beef (telopeptide-poor collagen), suggesting differences in molecular interactions and network formation between the two different collagen types. The authors concluded by saying that the modulation of chicken collagen by NaCl and soy proteins enabled them to produce functional chicken collagen films. Currently beef collagen is the primary source for co-extruded casings. However,

one of the major suppliers has recently introduced chicken collagen and it is yet to be seen how it will be accepted by the market.

3. Conclusions and future prospects

This review compiles currently available information regarding aspects of co-extrusion collagen casing production and manufacturing, which are important in producing high quality sausages. Furthermore, information regarding factors affecting collagen characteristics and possible use of other collagen sources for co-extrusion purposes are discussed. The latter is especially important today as the cost of natural casings is rapidly rising and the demand for Kosher and Halal products puts pressure on non-pork collagen supply. In addition, sustainable animal production is of utmost importance today and valuating meat plant by-products, with no / low value; can increase sustainability of the whole meat production chain.

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CHAPTER 2

Properties of different poultry skins sources in relation to co-extruded sausage casings

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Abstract

Casings are an essential component in the transformation of comminuted meat into a finished sausage. Their strength and the texture of the ground meat determine the "bite" perception when eating a sausage. Traditionally, meat has been stuffed into natural casings, but alternatives, such as cellulose and co-extruded collagen casings are emerging. Bovine hide split collagen is the primary source for co-extruded casings. However, an increase in meat products consumption puts pressure on the supply of collagen casings, and therefore producers are searching for alternatives. In this study, the properties of chicken skin collagen preparations from four types of birds [fast-growing broilers (42 d), slower-growing broilers (56 d), broiler breeders (52 wk), and laying hens (100 wk)] were investigated by biochemical, and physical analyses to obtain properties important in designing new dispersions for co-extrusion. SDS-PAGE, rheology, DSC and TNBS showed little difference in parameters between the different chicken types. However, after salt precipitation, creating strong films from the broiler breeder and laying hen skins' dispersions was not possible. Creating films was possible with the dispersions of fast and slower-growing broiler skins, particularly after precipitation with saturated NaCl.

In conclusion, chicken skin collagen from slower and fast growing broilers have the potential of being a suitable collagen source for the co-extrusion process. Overall, it was feasible to form stronger films with broiler skins than with skins of broiler breeder and laying hens. This is important as the casings' strength dictates the initial sensory perception when eating a sausage.

1. Introduction

Sausages have an important place in our diet, due to their nutritional density, shelf life, unique texture, and flavor (Savic and Savic, 2016). In the process of sausage making, a casing is an essential component in the transformation of comminuted meat into a finished product (Osburn, 2000; Suurs and Barbut, 2020; Barbut et al, 2020). Traditionally, sausage meat has been stuffed into intestines derived from pigs, sheep, and cattle, known as natural casings (Adzaly *et al.*, 2015; Savic and Savic, 2016). However, the limited availability of natural casings, the challenges of working with them, and their relatively high price enhanced the development of alternatives, such as cellulose casings, manufactured collagen, and the more recently developed co-extrusion casings (Barbut, 2010). Co-extrusion technology eliminates the intermediate stages of preparing and storing pre-made casings (Suurs and Barbut, 2020). Unlike traditional sausage production, where casings are stuffed with meat, co-extrusion introduces a thin layer of semi-liquid material that is extruded onto the meat batter as it is being discharged from the extrusion nozzle. This is later followed by the so called "gelled in place" to harden the sausage casing. Currently, beef collagen is the primary source for this semi-liquid material. It is a by-product of the meat packing industry; obtained from the skins of slaughtered beef cattle (Hoogenkamp *et al.*, 2015; Suurs and Barbut, 2020). However, as meat and meat products consumption have significantly increased over the past two decades, there has been a shortage of natural collagen casings, which has been paralleled by a significant price increase (Oechsle, 2016). Consequently, collagen producers are searching for alternative sources. One of these include poultry by-products, such as chicken skins, feet and bones, as currently mostly turned into low value products (Arunmozhivarman *et al.*, 2017). Oechsle *et al.* (2016) indicated that chicken skin could be an alternative source. Studies on collagen from avian species by Liu, Lin, & Chen (2001) and Lin & Liu (2006) reported that the molecular structure of collagen from avian species is different from mammalian species. This is obviously a species effect, but may also be partially related to the age of the animal. For example, beef cattle are processed at 18 – 40 months (Miller *et al.* 1983; Burke, 1980), whereas broilers are processed at 5 – 6 weeks of age. The animal's age influences the collagen structure, and as the animal grows older, the degree of covalent crosslinks increases, due to lysyl oxidase-initiated crosslinking (Oechsle, 2016; Noorzai, 2020). Collagen originating from younger animals is easily solubilized during the first separation stage in alkaline environment (Noorzai, 2020) because the crosslinks are not so stable, but acid soluble. Consequently, beef collagen from older animals has more crosslinks than broiler collagen and this may considerably affect the applicability of the collagen for casing production. It can be hypothesized that collagen originating from older poultry, such as broiler breeders (average 52 weeks old) and laying hens (average 100 weeks old), may be more suitable for casing production than collagen from young broiler chickens (Gojkovic *et al.*, 2014).

However, there seems to be a limit to the age of animals that can be used for collagen casing production, as the stiffness of collagen fibers continues to increase with more crosslinking, and this eventually results in higher brittleness and a lower tensile strength of the fibers (Varnali, 2002).

Oechsle *et al.* (2016), evaluated the application of various chicken collagen samples (chicken skin and bone collagen) within the co-extrusion process, and reported that broiler skin collagen was the most suitable source. However, there is no scientific data indicating whether commercially available skins, of different poultry species, are suitable to serve as a collagen source for co-extruded sausage casings. Collagen obtained from various poultry species may feature different gel properties, yielding films with different functionalities (e.g., stretch ability, appearance, bite). Different gel properties could also lead to differences in performance. Therefore, the goal of this study was to explore whether chicken skins, obtained from chickens of varying ages, could be used for the production of high-quality co-extrusion casings. The experiment compared the biochemical composition of skin collagen from fast and slower-growing broilers, broiler breeders and laying hens, employing both biochemical and physical measurements. The overall aim of this study was to determine essential properties required for selecting potential poultry collagen for the co-extrusion process.

2. Materials and methods

Chicken breast skin from different age birds: fast-growing broiler chickens (**FBC**, 42 days old), slower-growing broiler chickens (**SBC**, 56 days old), broiler breeders (**BB**, 52 weeks old) and laying hens (**LH**, 100 weeks old) were collected from local processing plants in Germany and The Netherlands. The dermis and epidermis were used for preparing the dispersions. Breast skin (10 kg) was collected of each age group, and then stored at -18°C until further processing.

2.1 Collagen isolation

A multistep process was used to obtain the dispersions prior to determining their physical and chemical properties. Sample preparation and pre-treatment were performed according to Munasinghe *et al.* (2015) with slight modifications. Briefly, chicken skins were thawed and cut into small pieces (0.5 x 0.5 cm). Non-collagenous proteins were removed by adding 0.1M NaOH (Boomlab, Meppel, The Netherlands) at a sample/solution ratio of 1:6 (w/v - based on the wet weight of the original sample). The mixtures were shaken for 24 h at 4°C. NaOH was then removed as samples were washed with distilled water and filtered (2.5mm mesh) until neutral pH was reached. Fat was removed by adding 10% butanol (Chemlab, Zedelgem, Belgium) at a sample/ solution ratio of 1:6

(w/v). The mixtures were shaken for 24 h, at 4°C. Butanol was then removed as samples were washed five times with distilled water and filtered (2.5mm mesh). The defatted samples were swollen in 0.5M acetic acid (Supelco, Zwijndrecht, The Netherlands) for 24 h at a sample/ solution ratio of 1:6 (w/v - based on the wet weight of the original sample), at 4°C. The skin samples were collected by filtering with a fine sieve (2.5mm mesh). Subsequently, the collected acetic acid was added to the mixture whereby the addition was calculated based on the protein content of the raw material. This was done to obtain a dispersion with a protein content of 3.5 to 4% protein. The samples were homogenized in a food processor (UMC5 Stephan Food Processing Machinery, Hameln, Germany) for 2 min at 1500 rpm under vacuum for FBC and SBC. BB and LH samples were homogenized for 3 min at 1500 rpm and 1 min at 3000 rpm under vacuum, as these samples were more viscous. The temperature of the mixture was kept below 20°C throughout the whole procedure. As a final step, the mixture was sieved (2.5mm mesh). The dispersions were stored at 4°C for 2 days until further analysis. For each chicken source, four dispersions (replicates) were produced, whereby on day 1 two dispersions of each chicken source were prepared and on day 2 the next two dispersions.

2.2 Protein and moisture content of raw materials

Protein content in the skin samples was determined by the Kjeldahl method using a conversion factor N of 6.25 (NEN-EN-ISO 8968-1). Dry matter content (NEN-ISO 1442) was determined by using ~2.5 g homogenized skin samples that were dried (103°C) for 16hr. Both analyses were done by a commercial laboratory. The four different skin types were evaluated in duplicates.

2.3 Protein and moisture content of chicken collagen dispersions

Dry matter content of the collagen dispersions was determined by lyophilization (Scan-vac, Coolsafe 55-4, LaboGene, Denmark), where approximately 25g were lyophilized for 72h, after which the amount was weighed, and the dry matter content calculated.

Protein content of the dispersions was measured by the Lowry method (Lowry *et al.*, 1951). In short, 50 mg of lyophilized chicken dispersions were incubated in 2.5U papain (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 1 ml digestion buffer (50mM $NaPO_4$, 2mM cysteine, 2mM EDTA, pH 6.5) for 16 h at 65°C. The digested samples were centrifuged (5 min; 13,000g) and supernatants used for protein measurement. A calibration curve was made, using BSA (0 to 250 ng/ml) and a blanc with only papain digestion buffer (50mM $NaPO_4$, 2mM cysteine, 2mM EDTA, pH 6.5). The reaction that occurs has a characteristic blue color with A_{max} at 750 nm.

2.4 Film preparation

For evaluation of the dispersions for film forming and textural abilities, films were made by placing 4g of the dispersions onto plastic sheets, which were then covered with a second sheet. A round mold with a raised edge of 0.3 mm was used to form 0.3 mm thick circles, with a diameter of 90 mm. Later, salt solutions were added to allow collagen to precipitate and to form a firm film. From each of the four replicates per skin type (FBC, SBC, BB, LH), six films were made with 24% NaCl and six were made with 40% K_2HPO_4 solution. In total 24 films per skin and salt type were produced and evaluated. Note that both NaCl and K_2HPO_4 are used in industry to harden co-extruded beef collagen casings.

2.5 SDS-PAGE analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to analyze the molecular weight of the protein chains in the collagen dispersions, and to assess the presence of other collagen types or collagen breakdown products. The method was adopted from Faraj *et al.* (2011). Briefly, 2.5mg of collagen was suspended in 150µl sample buffer and heated for 10 min at 70°C. Samples and controls were loaded on an 8% (w/v) polyacrylamide gel. After running at 150V, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue solution (R-250 (VWR International BV, Amsterdam**,** The Netherlands)**.** Each of the four skin type replicates (FBC, SBC, BB, LH) was used to produce a gel.

2.6 Thermal transition measurement

To study the thermal behavior of the chicken skin dispersions and the precipitated films Differential Scanning Calorimetry (DSC) measurements were performed. The endothermal transition of collagen was measured (DSC Q1000, TA Instruments, New Castle, DE, USA) equipped with a cooler (RCS40). Five to ten mg of collagen dispersion or precipitated film was hermetically sealed in a T_{zero} aluminum pan. The system was equilibrated at 1°C for 5 min. For the collagen dispersion, a temperature ramp was performed from 1 to 80°C, at 5°C/ min, while the precipitated collagen films were treated with a ramp of 1 to 100°C, at 5°C/ min. In both cases, an empty aluminum pan was used as the reference probe. Temperature at which helix to random coil transition of the collagen started (T_{onset}), temperature at which 50% of the collagen has unfolded (T_{peak}), and the value of denaturation enthalpy (ΔH) were determined using the DSC software (Universal Analysis 2000 (Version 4.5A), TA instruments). The four replicates of each skin type were measured in duplicate.

2.7 Rheology

To investigate differences in flow behavior between the different raw materials, which could indicate the applicability of the dispersions on a co-extrusion system, rheology measurements were performed based on Oechsle *et al.* (2016), with slight modifica-

tions. Experiments were conducted using an oscillatory rheometer type AR2000 (TA Instruments, New Castle, USA) equipped with Peltier plate and water bath. The collagen dispersions were analysed by oscillating measurements using a plate-plate geometry of 40mm diameter hard-anodised aluminium. Stress sweeps were performed at 1Hz to determine the linear viscoelastic range applying oscillatory stress from 0.01 to 1000Pa at 5°C, followed by frequency sweeps performed in the linear viscoelastic range applying 0.1% strain from 1 to 100 rad/s. The complex viscosity η^* was determined as a function of angular frequency ω by applying Eq. 1 (Macosko, 1994):

$$\eta^* = \left[\left(\frac{G''}{\omega}\right)^2 + \left(\frac{G'}{\omega}\right)^2\right]^{1/2} \quad \text{(Eq. 1)}$$

The power law relation of the complex viscosity η^* as a function of the angular frequency ω was used to calculate dynamic consistency index k^* and the dynamic power law factor n^* (Keogh and O'Kennedy, 1998) by applying Eq. 2.

$$\eta^* = k^* \, \omega^{n^* - 1} \quad \text{(Eq. 2)}$$

Temperature sweeps of the dispersion were performed by applying 0.1% strain from 5 to 60°C with ramp rate of 2°C/ min at 1Hz. The temperature at which G' (Pa) started to decrease as a measure of the helix to random coil transition was determined. The four replicates of each skin type were measured in duplicate.

2.8 Texture

Puncture force parameters were determined of the four replications per skin type (FBC, SBC, BB, LH), whereby per skin type six films prepared with 24% NaCl solution and six films prepared with 40% K_2HPO_4 solution were evaluated. The puncture force was done by fixing the films to a holder and using a spherical probe (P/5S, Stable Micro Systems, Surry, United Kingdom), attached to a texture analyzer (TA.XT2, Stable Micro Systems) equipped with a 5 kg load cell while employing a crosshead speed of 10mm/s. Maximum peak force and area under the peak were determined (Bourne, 1978).

2.9 Determination of primary amine group content

The concentration of primary amine groups present in the lyophilized chicken dispersions were determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS). The method was adopted from Buttafoco *et al.* (2006). Samples of 1.5mg were incubated for 30 min in an aqueous solution of 4% w/v $NaHCO_3$ (1mL). Then a solution of 0.5% w/v 2,4,6-trinitrobenzenesulfonic acid (TNBS) (1mL) in MilliQ water was added and the mixture was incubated at 40°C for 2h. After the addition of HCl (3mL, 6M), samples were hydrolyzed at 60°C for 90 min. The reaction mixture was diluted in a 96 well plate 1:1 with MilliQ water, mixed well and the absorbance was measured at 420nm using a spectrophotom-

eter (Bio-Tek, Bad Friedrichshall, Germany). The concentration of free amine groups was determined using a glycine calibration curve. The four replicates of each skin type were measured in triplicate.

2.10 Statistical analysis

Statistical analysis on all data was performed in Minitab Version 19 (Minitab Ltd., Coventry, UK). Data were checked for normality in both means and residuals. A general linear model was used for analysis of variance (ANOVA) for the thermal transition temperature, mechanical characterization, and determination of primary amine group content. The model used for the thermal transition temperature and primary amine group content was:

$$Y = \mu + \text{Skin type} + \text{Day} + e.$$

Where Y = dependent variable, μ = overall mean, Skin type = type of skin (FBC, SBC, BB, LH), Day = production day of the dispersion (1 or 2), e = residual error.

The model used for the mechanical characterization was:

$$Y = \mu + \text{Skin type} + \text{Day} + \text{Salt type} + \text{Salt type*Skin type} + \text{Day} + e.$$

Where Y = dependent variable, μ = overall mean, Skin type = type of skin (FBC, SBC, BB, LH), Day = production day of the dispersion, Salt type = type of salt (NaCl, K_2HPO_4), Salt type*Skin type = interaction between skin type and salt type, Day = production day of the dispersion (1 or 2), e = residual error. Data are presented as means ± SE. Means were compared, after correction for multiple comparisons, using the Bonferroni post-hoc test ($p<0.05$).

3. Results and discussion

3.1 Chemical characterization of raw materials

The aim of the research was to investigate whether skins of different avian sources and ages can be used to produce high quality collagen casings. To allow comparing their performance in the dispersions, skins were first analyzed for protein, and moisture content. The fat content was calculated based on the protein and moisture content, Protein content (w/ w) of FBC, SBC, BB and LH skins were 9.1%, 11.5%, 10.2% and 16.4%, respectively. On dry weight basis, these values translate to a protein content of 15%, 21%, 22% and 36%, respectively. Fat content (w/ w) of FBC, SBC, BB and LH skins were

calculated on 51%, 44%, 35% and 29%, respectively. On dry weight basis, these values translate to a fat content of 83%, 76%, 75% and 62%, respectively. Inconsistent results are found for the protein and fat content of chicken skins in literature. Munasinghe *et al.* (2014), reported protein and fat contents of 22.6% and 31.6%, respectively, on dry weight basis. Although the protein content is comparable to our FBC sample, the fat content was remarkably lower. Additionally, Bonifer *et al.* (1996), reported 9.1% protein and 37.2% fat in the fresh skins of 6 to 8 weeks old broilers. The protein content is in accordance with the 42-day old chicken skins in our study, however the fat content again much lower. Swatland *et al.* (2007) reported chicken skin to contain 45.1% fat and 6.6% protein. It is evident that results can vary quite a lot. This is explained by the fact that the chicken skin composition might be affected by several factors, such as age, dietary differences and location; e.g. back or breast skin (Bonifer et al, 1996). Often the body part and the age of the chickens are not mentioned in the literature. Based on the current findings it can be concluded that at least age is an important factor affecting protein concentration of the skin. Protein content of the older animals in our study (LH) was in the same range as found for bovine skin that is typically used for sausage casing production; i.e., 30% on dry weight basis, which also depends on the animal age (Noorzai *et al.*, 2019).

3.2 Chicken collagen dispersions

The dry matter content of the dispersions prepared from FBC and SBC skins differed from the BB and LH skins. The former had a higher dry matter content (12.83 ± 0.22% and 12.93 ± 1.13%, respectively). Compared to the BB and LH with lower dry matter ($p<0.00001$) (8.62 ± 1.31% and 10.84 ± 1.23%, respectively). Moreover, a significant difference ($p<0.05$) was found between the BB and LH dispersions. Differences in dry matter content are probably caused by the higher amount of acid addition to the BB and LH skins. Overall, the FBC and SBC collagen was already more swollen after soaking in acetic acid than the collagen of the BB and LH skins.

On the contrary, the protein content of FBC, SBC and LH freeze-dried skin samples was lower than the protein content of the BB samples (7.6 ± 1.4%, 7.4 ± 1.5%, 10.4 ± 2.8% vs 15.8 ± 2.6%, respectively, ($p<0.004$). It should be noted that the values are probably an underestimation of the actual protein content, because not all the gel was digested by papain. In addition, after centrifugation, some fat was floating on top of the suspension and the presence of fat in the samples could made protein extraction more difficult.

3.3 SDS-PAGE

The SDS-PAGE gels (Fig. 1) clearly show the presence of two distinctive bands: 100kD and 130kD, indicating α1(I)- and α2(I)-chain monomers of type I collagen (molecular mass of $\alpha_2 < \alpha_1$ with approximate mass ratio $\alpha_1 : \alpha_2 = 1:2$) (Gojkovic *et al.*, 2014).

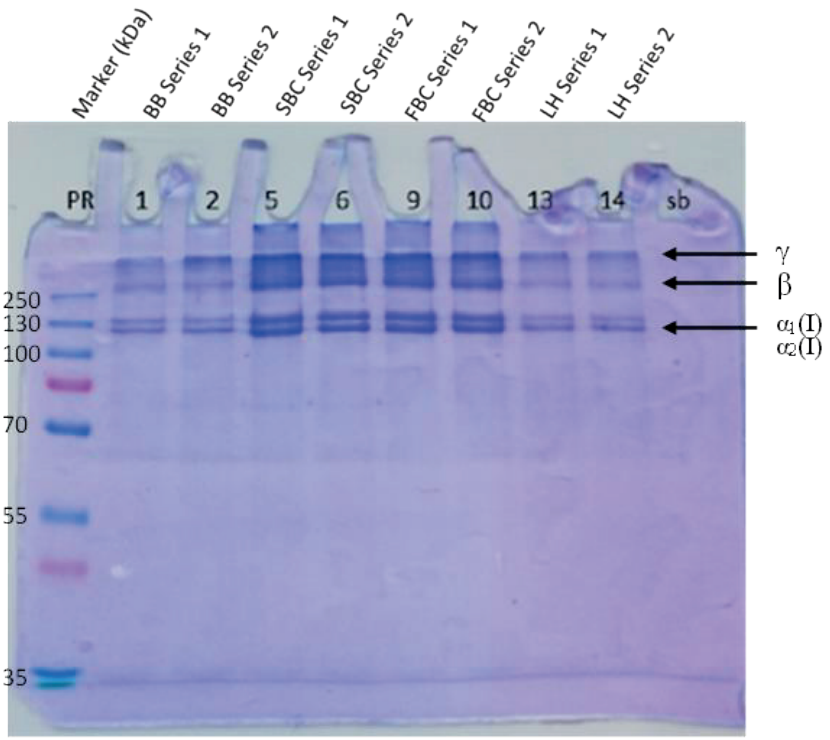

Figure 1. SDS-PAGE gel of the marker and of the dispersions of broiler breeder (BB), slower growing broiler chicken (SBC), fast growing broiler chicken (FBC) and laying hen (LH) indicating the presence of α1(I) and α2(I) bands which are particular for type I collagen. It should be noted that only a small fraction of the collagen is soluble and placed on the gel. Most of the collagen is insoluble, resulting in a pellet left in the sample buffer.

According to Abedin and Riemschneider (1984) chicken skin contains types I and III collagen. However, α1(III) bands were not detected on our gels. α1(III) collagen was expected at 138kDa (Oechsle *et al.*, 2016). The gels also clearly show a difference in contrast of the bands of SBC and FBC, in comparison with BB and LH. The BB and LH dispersions were likely more intra- and intermolecularly crosslinked than the FBC and SBC dispersions, as crosslinks increases with age (Oechsle *et al.* 2016; Pines *et al.* 1996; Yamauchi *et al.* 1988). Furthermore, the SDS-PAGE showed that no major denaturation of the collagen had occurred, due to the pretreatment of the skins, since there were no bands visible at the lower molecular weight.

3.4 Differential Scanning Calorimetry (DSC) of collagen dispersions and films

Measurements were made to study the thermal behavior of chicken skin dispersions from different ages. In the sausage making process, the collagen dispersion is converted

into a film by partial dehydration (precipitation) with a saturated salt (e.g., NaCl) solution, followed by crosslinking and finally a heating step to cook the sausage (Suurs and Barbut, 2020). During this transformation process, the collagen is transformed from a helical to a crystalline structure (Bianchi and Conio, 1967). During the heating step, it is important that the collagen does not change from the crystalline structure to the random coil phase, as in that phase the collagen can unfold (i.e., transformed to gelatin), and result in sausages with a sticky/mushy casing. This means that collagen dispersions and films need a high helix-to-coil transition temperature to prevent random coil formation, and this correlates with a higher stability at a high temperature environment (Miles *et al.*, 2005). In general, chicken dispersions investigated here showed higher T_{onset} temperatures (Table 1) compared to the commercial bovine collagen dispersions, studied by Barbut *et al.* (2020). The current study shows that T_{onset} (average 40.95°C), T_{peak} (average 44.02°C) and enthalpy (0.241 J/g) of the dispersions prepared from young chicken skins (SBC and FBC) were not significantly different from the dispersions made from old chicken skins (BB and LH; Table 1). T_{onset} values for bovine collagen dispersions ranged from 33.5 to 34.5°C (Barbut *et al.*, 2020).

The transition from a chicken collagen dispersion to a film by partial dehydration (exposure to 24% NaCl) resulted in a higher thermal stability for all dispersions. The increase is due to decreasing moisture content; i.e., during the salt dehydration step. This results in a compact structure formation, in which the fibers are closer to each other. T_{onset} of films prepared from the LH skins was 61.77°C. It was significantly higher (P=0.004) than the T_{onset} of the films prepared from SBC, FBC and BB skins (57.33°C, 56.08°C and 57.93°C, respectively). Barbut *et al.* (2020), also showed a significant increase in thermal stability of commercial bovine collagen dispersions that were transferred into collagen films by salt precipitation: endothermic peaks starting at 58.2 to 60.3°C, with maximum values at 63.9 to 65.3°C. This trend is in accordance with the current chicken collagen films. McPherson *et al.* (1986), suggested that stronger association of bovine collagen fiber structure is correlated with increased denaturation temperature.

The films prepared from skins of older chickens (BB and LH) clearly show two helix-to-coil-transitions, whereas the FBC and SBC films only show one transition (Table 1, $T_{onset\ 1}$ Fig. 2). The second transition, at approximately 70°C, for BB and LH (indicated by $T_{onset\ 2}$ Fig. 2), could be related to the lysine-aldehyde derived crosslinks that are replaced by mature crosslinks in the collagen of the aging the bird (Miles *et al.*, 2005). Given the higher thermal stability of chicken skin dispersions (ranging from 40.9 to 41.5°C) compared to bovine skin dispersions (ranging from 33.5 to 34.5°C), all four types of chicken skin sources should be acceptable for casing production. However, when considering the thermal stability of the collagen films, the films made of the LH skin is preferred over the others as it shows the highest thermal stability. Overall, we need to have a high

Table 1. Analysis of endothermic peaks from differential scanning calorimetry (DSC) thermograms. The four replicates of each chicken source [fast growing broiler chicken (FBC), slower growing broiler chicken (SBC), broiler breeder (BB) and laying hen (LH)] were determined in duplicate both as collagen dispersion (n=4 per type) and as dehydrated films (n=4 per type). Skins of fast growing broiler chicken (FBC), slower growing broiler chicken (SBC), broiler breeder (BB) and laying hen (LH) were measured (n=1 per type). Means ± standard deviation.

Type	Dispersion/ Film/Skin	T_{onset} (°C)	T_{peak} (°C)	Enthalpy ΔH (J/g)	T_{onset} (°C)	T_{peak} (°C)	Enthalpy ΔH (J/g)
FBC	Dispersion	41.02 ± 0.42	44.16 ± 0.67	0.232 ± 0.082	-	-	-
SBC	Dispersion	40.88 ± 0.33	43.87 ± 0.39	0.249 ± 0.127	-	-	-
BB	Dispersion	41.11 ± 0.32	45.28 ± 0.62	0.162 ± 0.103	-	-	-
LH	Dispersion	41.50 ± 0.25	44.52 ± 0.92	0.048 ± 0.025	-	-	-
P-value		0.42	0.37	0.12			
FBC	Film	56.08 ± 0.48 ^{y}	61.78 ± 1.62	0.455 ± 0.103 ^{x}	-	-	-
SBC	Film	57.33 ± 0.55 ^{y}	62.85 ± 0.96	0.379 ± 0.052 ^{x}	-	-	-
BB	Film	57.93 ± 2.13 ^{y}	62.15 ± 3.21	0.037 ± 0.005 ^{y}	69.72 ± 1.54	74.75 ± 1.82	0.282 ± 0.115
LH	Film	61.77 ± 1.78 ^{x}	64.61 ± 1.66	0.017 ± 0.007 ^{y}	70.12 ± 0.94	75.98 ± 1.56	0.110 ± 0.038
P-value		0.00	0.20	0.00			
FBC	Skin	61.64 ± 1.76	66.93 ± 0.83	7.231 ± 0.383	-	-	-
SBC	Skin	62.55 ± 0.49	65.44 ± 1.89	2.567 ± 0.724	-	-	-
BB	Skin	64.43 ± 0.03	66.42 ± 0.76	5.875 ± 2.775	-	-	-
LH	Skin	65.75 ± 1.32	68.39 ± 2.04	3.455 ± 4.235	-	-	-

T_{onset} = onset temperature; T_{peak} = temperature at 50% heat flow; ΔH = total enthalpy changes in melting of collagen.
^{a,b,x,y} = Means within a column and dispersion/ film not sharing a common superscript differ (P<0.05).
- = Not available

helix-to-coil transition temperature to prevent random coil formation of the collagen film in the sausage production process.

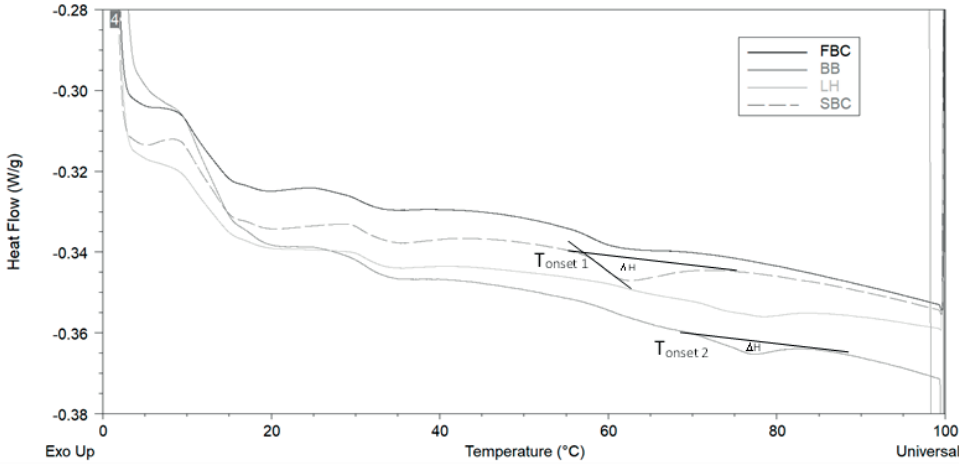

Figure 2. Differential scanning calorimetry showing representative helix-to-coil transition of chicken collagen dispersions obtained from FBC (fast growing broiler chicken), SBC (slower growing broiler chicken), BB (broiler breeder) and LH (laying hen) skins. . Heating rate 5°C/ min, indicating T_{onset1} for a dispersion prepared from SBC skins, and T_{onset2} for a dispersion prepared from BB skins.

3.5 Rheology

3.5.1 Temperature profiles

In the sausage making process, the dehydration of co-extruded collagen follows a heating step to cook the sausage. Consequently, it is important to measure the collagen melting temperature. The rheological tests showed that between 40°C and 45°C all dispersions started to display a rapid decrease in elasticity (Fig. 3). This temperature range was not found for bovine collagen dispersions where a rapid decrease was reported between 30 and 45°C (Barbut *et al.*, 2020). This may be attributed to a two times higher lysine content in chicken skin collagen compared to bovine skin collagen (Gojkovic *et al.*, 2014). Lysine provides thermal stability as Lysine – Glycine –Y links are involved in side-chain interactions (Brodsky and Persikov, 2005; Gojkovic *et al.*, 2014).

The rheological transitions observed for the dispersions (loss of elasticity, Fig. 3) were in the range of the onset temperatures measured by DSC. This means that all four chicken skin sources appear to be suitable for making casings, as indicated earlier. The elasticity loss of the dispersions at 40 to 45°C showed that the collagen in these dispersions were significantly modified by the preparation procedure (NaOH treatment and acidification),

as all chicken skins (starting material) showed denaturation temperatures above 60°C (Table 1).

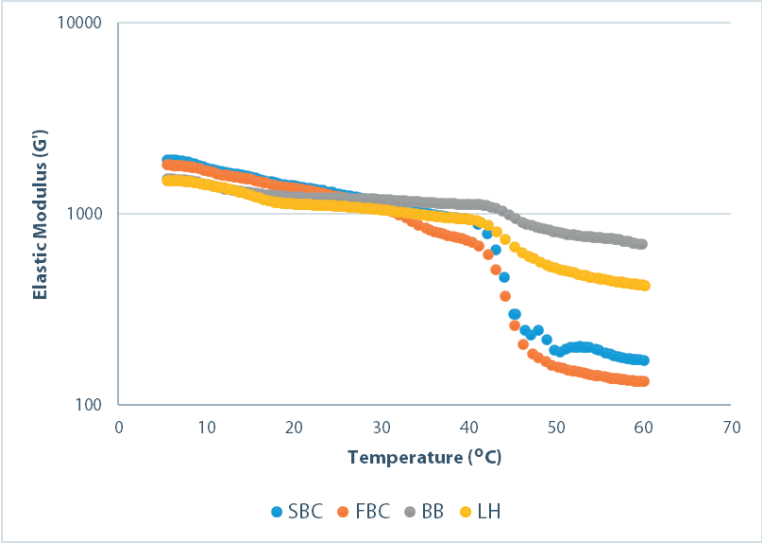

Figure 3. Elastic modulus (5 to 60°C at 2°C/ min) of four chicken collagen dispersions varying in age: SBC (slower growing broiler chicken), FBC (fast growing broiler chicken), BB (broiler breeder), and LH (laying hen), showing a rapid decrease in elasticity for all dispersions between 40°C and 45°C.

3.5.2 Complex viscosity

Viscosity measurements can provide valuable information (e.g., flow properties of dispersions) to the sausage producers using a co-extrusion system. Figure 4 shows that all dispersions show shear-thinning behavior following a power-law model, and that they all reveal the same complex viscosity. The complex viscosity η^* is a measure of the total resistance to flow as a function of angular frequency (ω), so the higher the value for the complex viscosity, the more resistance the material is to flow. The expectation was that the complex viscosity of the collagen dispersions made from the older chicken skins (BB and LH) would be higher than those from young chickens (SBC and FBC). This is because the presence of intra- and intermolecular crosslinks in the BB and LH dispersions, making them more resistant to flow. Oechsle *et al.* (2014) found contradictory results when comparing a highly crosslinked native collagen with a telopeptide-poor collagen. The former showing a lower η than telopeptide-poor collagen without crosslinks present. They attributed this to the presence of crosslinks in the material.

Table 2 shows the dynamic consistency index (k^*) and dynamic power law factor (n^*) for the chicken collagen dispersions. The k^* describes the consistency of the dispersion, where a higher value means higher consistency of the material. Table 2 shows

that dispersions made from the older chicken skins were lower than the younger ones. Dispersions made from BB and LH skins showed lower k^* values, probably caused by the higher number of crosslinks in the older skin. These crosslinks are affecting with the rheological measurements, thereby probably underestimating the value (Oechsle *et al.* 2016). Oechsle *et al.* (2016), also measured a dispersion made from chicken skin and reported a consistency value of 606 Pa s^{n^*}. Their collagen extraction process and the higher dispersions' protein content probably resulted in the large difference compared to current findings.

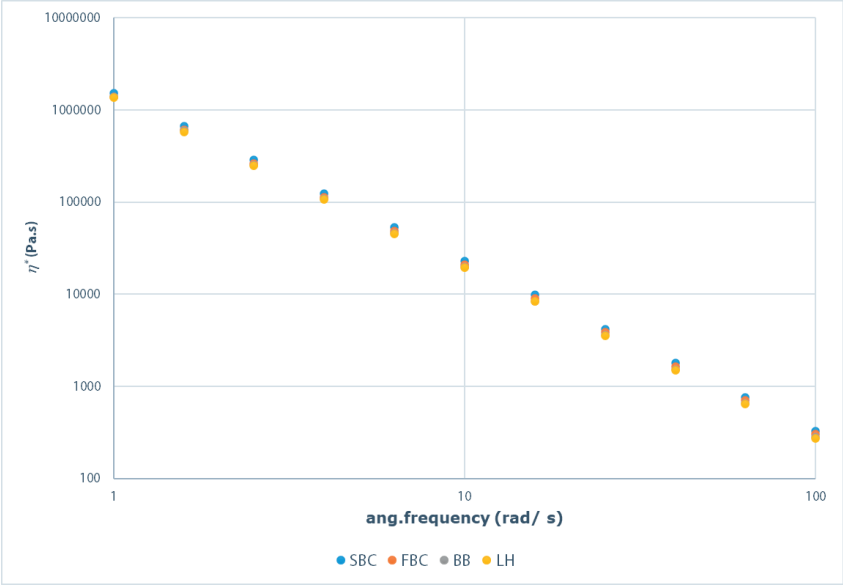

Figure 4. Complex viscosity η^* calculated from Eq. 1 of chicken collagen dispersions of SBC (slower growing broiler chicken), FBC (fast growing broiler chicken), BB (broiler breeder) and LH (laying hen) as a function of the angular frequency ω (n=4).

The dynamic power law factor n^* indicates shear-thinning ($n^* < 1$), shear-thickening ($n^* > 1$) or Newtonian ($n^* = 1$) behaviour. Shear-thinning behaviour means that n^* decreases with increasing values of ω. Viscoelastic systems are intermediate between 0 and 1 (Keogh *et al.*, 1998). Table 2 shows that n^* values for all four dispersions were similar and almost 0, which means that they have almost only an elastic components. Oechsle *et al.* (2016), reported a n^* value of 0.13 in their experimental chicken skin dispersion. This means that their dispersion also had some viscous components. Unfortunately, no data is present regarding the dynamic consistency index k^* and n^* for commercial bovine collagen dispersions. This makes it difficult to compare current results with most used (by the industry) bovine collagen dispersions, and to judge whether the chicken collagen dispersions have similar flow properties.

Table 2. Dynamic consistency index (k^*) and dynamic power law factor (n^*) calculated from Eq 2. by making a double logaritmic plot of complex viscosity (η^*) versus angular frequency (ω) of collagen dispersions made from FBC (fast growing broiler chicken), SBC (slower growing broiler chicken), BB (broiler breeder), and LH (laying hen).

Collagen dispersion	k^* (Pa s^{n^*})	n^*
Fast growing broiler chicken	41	0.01
Slower growing broiler chicken	44	0.01
Broiler breeder	34	0.01
Laying hen	35	0.01

3.6 Texture

An important aspect of the collagen dispersion is the ability to form a strong film. A strong film is desired because the final goal is to co-extrude the dispersion onto meat dough to form a casing. The strength of the casing determines, together with the texture of the ground meat, the sensory perception "bite" when eating a sausage. Puncture tests were used as a tool to evaluate the force and energy required to puncture the films after exposing the dispersion to 24% NaCl or 40% K_2HPO_4 (as is also done under industrial settings). The general observations was that the SBC and FBC dispersions resulted in fairly firm and manageable films that performed well in the puncture test. However, the films made from BB and LH dispersions were difficult to handle (mushy texture) making puncture measurements difficult to execute (Fig. 5).

Figure 5. Images of films prepared from dispersions originating from broiler breeder skins (A) and from fast growing broiler chickens (B) precipitated with 24% NaCl. The figure shows the resulting semi transparent film (image A), and opaque (B). Note: an opaque appearance is associated with a salt-precipitated dispersion forming a strong film.

No significant interaction was found between skin type and salt type (Fig. 6) and no significant difference in puncture force was found between the different skins (Fig. 6). A significant difference ($p<0.05$) was found between the different salt types (NaCl or K_2HPO_4), used for precipitating the dispersions. No significant difference in total energy required to puncture the films was found between the different salts and skin types (Fig. 7).

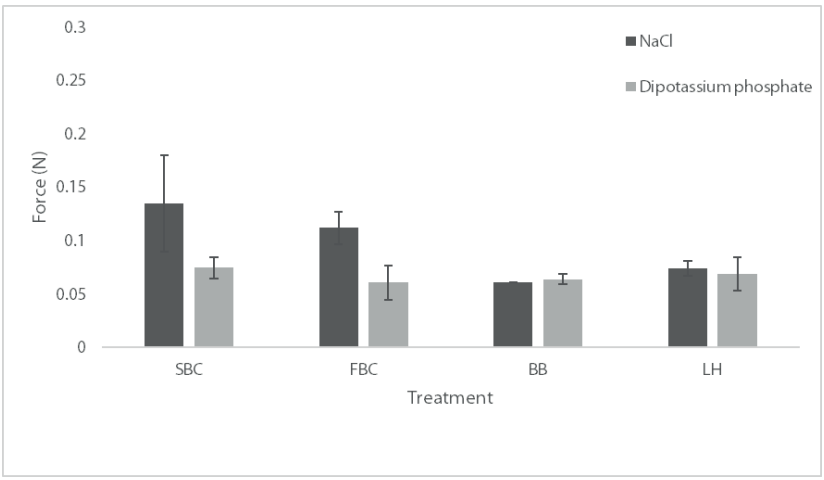

Figure 6. Ultimate force (N) to puncture films made from chicken collagen dispersions treated with 24% NaCl or 40% K_2HPO_4 solutions for SBC (slower growing broiler chicken), FBC (fast growing broiler chicken), BB (broiler breeder) and LH (laying hen); n=4. Bars represent means ± standard deviations.

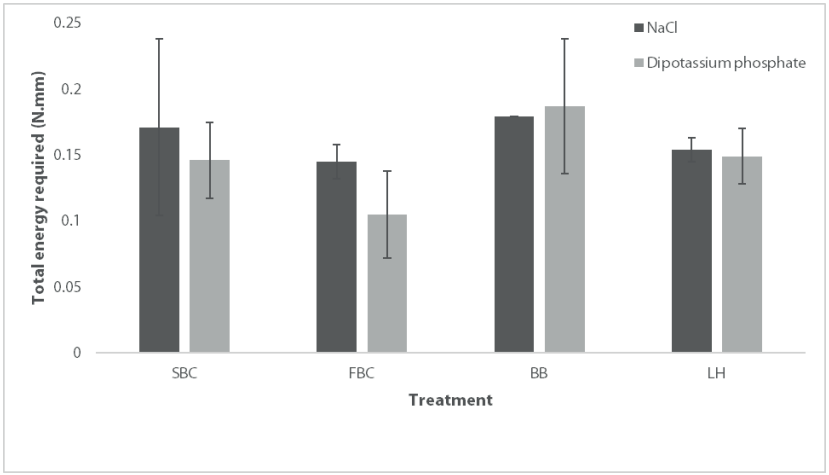

Figure 7. Total energy required (N.mm) to puncture films made from chicken collagen dispersions treated with 24% NaCl or 40% K_2HPO_4 solutions for SBC (slower growing broiler chicken), FBC (fast growing broiler chicken), BB (broiler breeder) and LH (laying hen) n=4. Bars represent means ± standard deviations.

Munasinghe *et al.* (2015) also extracted collagen from broiler skins and evaluated the strength of these films. They reported a higher film strength, ranging from 5 to 158N compared to the current study. However, their films were prepared by casting and drying instead of precipitation (note: drying results in about at least 4x higher dry content level). Moreover, collagen was crosslinked with glutaraldehyde; i.e., known as a very efficient crosslinker that also raises strength of protein films/ gels. Barbut *et al.* (2020) evaluated five commercial bovine collagen dispersions by preparing films by using a stainless steel roller with a recess of 0.50 mm, followed by partial dehydration in a 30% NaCl solution. They found that the amount of work to break the films (puncture test) was approximately 16 times higher compared to the values found in the current study. The difference might have been caused by several factors. First, the protein concentration (3.5 to 4.0% vs. 4.0 to 5.0% in their study). Second, the preparation procedure of the dispersions; e.g., small scale equipment versus large scale industrial preparations, where homogeneity of the dispersions is more difficult to obtain in small scale lab equipment. Third, the origin of the material and therefore differences in collagen structure of the raw materials; bovine versus chicken collagen. Gojkovic *et al.* (2014) investigated the amino acid composition of chicken skin collagen and beef skin collagen of animals at different ages. They found that bovine collagen had a higher glycine content (13.7 g/ kg versus 6.2 g/ kg of protein for skin of a 5 year old hen) and proline (10.31 g/ kg versus 4.2 g/ kg of protein for skin of a 5 year old hen). This contributes to a higher rigidity of the collagen coils and fibril strength, because Glycine –Proline -Y units were most stabilizing in the triple helix. A fourth factor could be the thickness of the bovine films which was ~ 1.5 times higher compared to our chicken films. Based on our texture findings, young chicken skins (SBC and FBC) are preferred over old chicken skins (BB and LH) as a source for making co-extrusion casings. Optimization of the skin pretreatment procedure is important in obtaining high quality dispersions and producing strong casings. This can include an enzyme solubilisation method for extracting collagen from mature tissue as described by Noorzai *et al.* (2019).

3.7 Evaluating the number of crosslinkable groups in chicken collagen dispersions

In the co-extrusion process, casings are crosslinked by applying liquid smoke, which contains aldehydes, reacting with the collagen free amine groups. The more free amine groups, the stronger the casing will eventually be. The number of crosslinkable groups was determined by assaying the free amine groups using a trinitrobenzene sulfonic acid (TNBS) assay. Data are expressed as the average values of nmol free amine groups per mg protein (Fig. 8). No significant differences in free amine groups were found between skin types, although it was expected to find higher numbers for the SBC and FBC chicken skins, as with raising collagen age there will be a higher degree of crosslinking (Gojkovic *et al.*, 2014). The reason that no significant difference was found may be due

to: a) the pretreatment procedure with NaOH swelling resulted in cleavages of the collagen structure, b) the dispersions contained fat, which was not completely removed in the pretreatment, c) the age difference between the FBC and SBC versus BB and LH was not enough to show any significant changes in crosslinking. In a chicken, skin integrity is dependent on the collagen content (Granot et al, 1991a,b) and crosslinking (Ramshaw et al, 1986). Pines *et al.* (1996) showed that up to 49 days of age no mature stable crosslinks such as pyridinoline or deoxypyridinoline could be detected, and only negligible amount of histidinohydroxylysinenorleucine (**HHL**) was detected in male and female chicken breast skin. In any case, there is a possibility that the lifespan of BB and LH chickens is not enough to develop these mature crosslinks. However, optimization of the pretreatment procedure would be necessary to get the maximum value out of these raw materials.

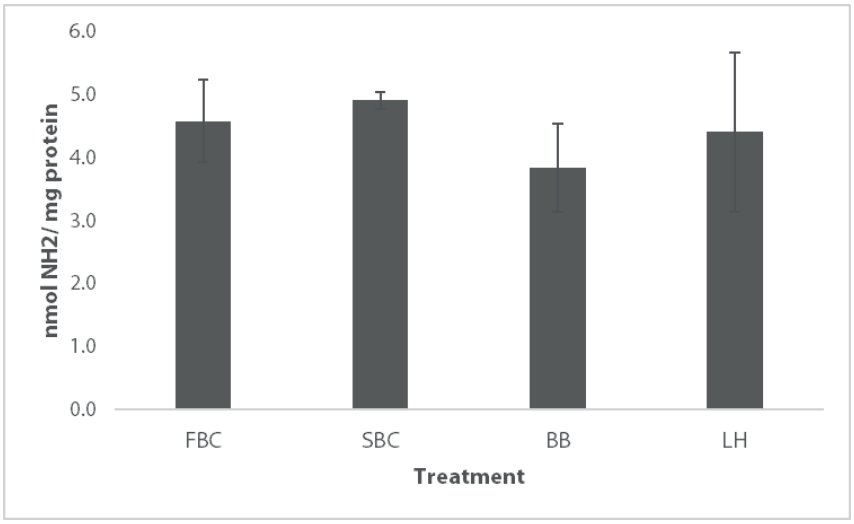

Figure 8. The number of crosslinkable groups present in chicken collagen dispersions prepared from skins of FBC (fast growing broiler chicken), SBC (slower growing broiler chicken), BB (broiler breeder) and LH (laying hen) n=4. Bars represent means ± standard deviations

4. Conclusions

Dispersions and associated films suitable for co-extrusion application should have a number of properties (Table 3). In the evaluation of the dispersions and the films, the emphasis was on the performance, assessed by texture measurements, as a casing dictates the initial sensory perception when eating a sausage. Based on the current study, chicken skin collagen from slower and fast growing broilers have the potential of being a suitable source of collagen for the co-extrusion process. As it is better possible to

form firmer films with young broiler chicken skins than with old chicken skins of broiler breeder and laying hens.

Table 3. Properties of dispersions and films per skin type [FBC (fast growing broiler chicken), SBC (slower growing broiler chicken), BB (broiler breeder) and LH (laying hen)] and their performance with respect to co-extrusion process and/ or final product

Measurement	Properties in relation to co-extrusion process/ final product	Fast growing broiler skins	Slower growing broiler skins	Broiler breeder skins	Laying hen skins
SDS-PAGE	Type I collagen	+	+	+	+
DSC	Helix-to-coil temperature	+	+	+	++
Rheology	Extrudability	+/-	+/-	+/-	+/-
Texture	Casing strength	+	+	-	-

+/ - = property of the dispersions/ films meets the criteria

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CHAPTER 3

3

Effects of broiler weight and strain on skin collagen characteristics and their applicability for co-extruded sausage casings

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Kudakwashe Farawu
Willeke F. Daamen
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Food Struct: 2023; 35: 100305

Abstract

For centuries people around the world enjoyed traditional sausages made from meat stuffed into natural casings. An alternative new technology is to extrude collagen gel, originating from bovine hides, directly onto the product and later cross-link it. Collagen producers are searching for other sources and consequently they are interested in factors influencing extracted collagen quality. One of the alternative sources is chicken skin, where extracted collagen properties have been shown to be influenced by the age of the chickens. In this study, the biochemical and physical properties of chicken skin collagen preparations from two different broiler strains (slow and fast-growing) and two different weights (1.6 and 2.2 kg) were investigated. Rheological measurements showed for all dispersions a decrease in elasticity at 40°C. Differential Scanning Calorimetry (DSC) measurements of the dispersions showed T_{onset} ranging from 38.7 – 39.1°C. After salt precipitation, the T_{onset} increased to 50.1 – 55.9°C. Mechanical strength of the films from fast and slow growing chickens ranged from 63 to 67 KPa and 53 to 57 KPa, respectively. Considering the biochemical and physical properties, all four chicken collagen dispersions have the potential of being a suitable collagen source for the co-extrusion process of sausages.

1. Introduction

Processed intestines from pigs, sheep, and cattle, which can be considered as by-products of the livestock industry, are used as edible natural casings to produce sausages (Wijnker, 2009). Sausage casings play important functional roles from the moment of stuffing right up until the consumer eats the product. The selection of a casing is critical, as it not only influences the integrity, size, and shape of the sausage, but also assists in the conversion of soft meat batter into the desired sausage product during cooking (Savic & Savic, 2016). Casings are designed to accommodate sausage manufacture's quality and processing needs. The ideal collagen sausage casings should provide sufficient strength to undergo processing, while remaining tender enough during consumption (Miller, 1983). Natural casings are perceived to have the highest quality and are considered as the golden standard for sausage casings; i.e., this is due to certain characteristics known as: bite, knack, snap and elasticity (Escoubas *et al.*, 2010; Savic and Savic, 2016). However, producers are searching for alternatives, because of the rising costs and availability of sheep and hog intestines as well as an increasing need for kosher/ halal products (Oechsle, 2016). An alternative casing material is collagen gel which, in combination with co-extrusion technology, can be applied directly onto a stream of meat exiting the stuffer's horn, and later splitting the meat rope into individual sausage links (Suurs *et al.*, 2022). Co-extruded casings are produced with a collagen gel, consisting of fibrous and soluble collagenous material (Barbut *et al.*, 2020). Currently, this collagen gel is particularly originating from bovine hides. However, alternative sources, like chicken skin collagen, have also been investigated for their application in co-extrusion technology (Oechsle *et al.*, 2016). Before application is possible, factors that influence collagen quality need to be investigated. Suurs *et al.* (2022) showed that chicken age is a factor that influences the collagen gel quality for co-extrusion application. Particularly the skins of young broiler chickens appeared to be suitable as a source for collagen gel for co-extrusion. However, within young broiler chickens two other important factors, such as strain and body weight, may influence the collagen properties. The aim of this study was to investigate effects of two broiler strains (slow and fast growing) at two different body weights (1.6 and 2.2 kg) on skin collagen quality, by focusing on its application for co-extruded sausage casings.

2. Materials and methods

2.1 Experimental design

The experiment was setup as a 2 x 2 factorial design with two broiler chicken strains (fast growing [**FBC]** and slow growing [**SBC]**) and two different body weights (1.6 and

2.2 kg), resulting in 4 treatments: FBC_1.6kg, FBC_2.2kg, SBC_1.6kg and SBC_2.2kg. The skins of fast growing (Ross 308) and slow growing (Hubbard JA 757) broiler chickens were obtained from a study described by Van der Eijk *et al.* (2022). Skin samples of 5 fast-growing chickens were randomly taken at day 28 (body weight 1.6 kg) and 10 skin samples at day 37 (body weight 2.2 kg). Skin samples of 5 slow growing chickens were randomly taken at day 37 (body weight 1.6 kg) and 10 taken at day 50 (body weight 2.2 kg). The whole skin (breast and neck) of the chickens was collected, followed by feather removal (manual plucking), after which the skins were stored as a pooled sample at -18°C, until further processing. No measures were taken to remove the subcutaneous fat. For the preparation of the collagen dispersions, both the dermis and epidermis were used. The pooled skin sample of each chicken source was divided into three portions so that three dispersions (n=3) could be prepared from scratch.

2.2 Pretreatment of the skins

Dispersions were prepared according to a multistep process from Munasinghe *et al.* (2014) with slight modifications. Next, they were analyzed for their physical and chemical properties (Fig. 1). Briefly, chicken skins were thawed, rinsed with tap water to remove debris, and cut into small pieces (5 x 5 mm). Skins were incubated in 0.1 M NaOH (Boomlab, Meppel, The Netherlands) at a sample/ NaOH ratio of 1:6 (w/v - based on the wet weight of the original sample). The mixtures were stirred continually for 5 h on a shaker at 4°C. NaOH was replaced every 2 h, and at the end it was removed by washing with distilled water and filtering (2.5 mm mesh) until neutral pH was reached. Thereafter, fat was removed by adding 10% butanol (Chemlab, Zedelgem, Belgium) at a sample/ butanol ratio of 1:6 (w/v – based on the weight of the swollen chicken pieces). The mixtures were stirred continually for 24 h at 4°C and the solvent solution was renewed after 12 h. At the end, butanol was removed by washing with distilled water and filtering. The defatted skin samples were soaked in 0.1 M HCl (Boomlab, Meppel, The Netherlands) with a sample/ HCl ratio of 1:6 (w/v). The mixture was stirred continually for 24 h at 4°C on a shaker, and filtered, followed by rinsing with distilled water until the pH of the wash water reached neutral pH.

2.3 Collagen extraction – preparation of soluble/ insoluble collagen dispersions

The pretreated samples were swollen in 0.5 M acetic acid (Supelco, Zwijndrecht, The Netherlands) for 48 h at a sample/ acetic acid ratio of 2:1 (w/v - based on the weight of the sample after pretreatment) at 4°C. Samples were then homogenized (JAP blender, Fusionesco, Q-Blend, 1500 W, The Netherlands) until a homogenous collagen dispersion was obtained.

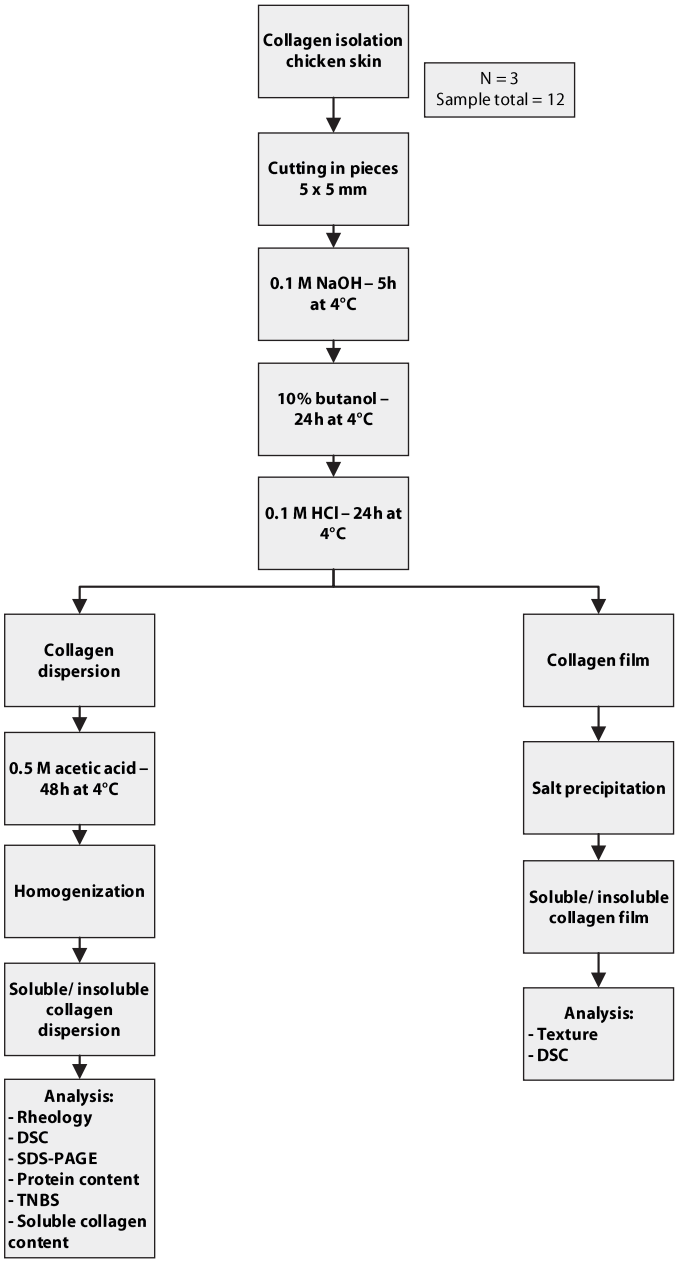

Figure 1. Flow chart of the extraction methods used to obtain collagen dispersions and films, including an overview of the methods to analyze the dispersions and films. The analysis methods include Differential Scanning Calorimetry (**DSC**), Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (**SDS-PAGE**), and 2,4,6-trinitrobenzenesulfonic acid (**TNBS**)

2.4 Collagen extraction – preparation of soluble collagen

Additional samples, from the fast and slow growing broilers with the heaviest weight (2.2 kg), were prepared to obtain an indication of the amount of soluble collagen in these dispersions. Samples were obtained in the same way as described earlier, but after swelling in 0.5 M acetic acid, the skin material was filtered (2.5 mm mesh), while the filtrate was collected and precipitated to obtain soluble collagen. Precipitation was done by adding NaCl crystals *(*Boomlab, Meppel, The Netherlands) until a final concentration of 2.6 M was reached. The solution was stirred and left for 60 h at 4°C. The precipitates were collected by centrifuging (Thermo Scientific, IEC, CL10, Fixed Angle Rotor F-G1) at 3,700 g for 30 min and supernatant was discarded. The resulting pellet was dialyzed (Ø 17.5 mm, molecular weight cutoff at 14 kDa; Medicell, London, United Kingdom) against 0.1 M acetic acid for 48 h.

2.5 Protein and moisture content of chicken collagen dispersions

Lyophilization (LP-03, IlShinBioBase Europe, Ede, The Netherlands) of the dispersions was done to determine the dry matter content of the collagen dispersions. Approximately 25 g wet material was lyophilized, followed by weighing of the dry material, and calculating the dry matter content.

Protein content of the lyophilized chicken dispersions was assessed (Lowry *et al.*, 1951). Incubation of 10 mg of lyophilized chicken dispersions in 5U papain (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 1 ml digestion buffer (50 mM $NaPO_4$, 2 mM cysteine, 2 mM ethylenediaminetetraacetic acid (**EDTA**, pH 6.5; Merck, Amsterdam, The Netherlands) was done for 16 h at 65°C. Followed by centrifugation (5 min; 13,000 g) of the digested samples. The supernatants were used for protein measurements. Bovine serum albumin (**BSA**) (0 to 250 ng/ml) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and a blank with only papain digestion buffer (50 mM $NaPO_4$, 2 mM cysteine, 2 mM EDTA, pH 6.5; Merck) were used to make a calibration curve. The blue color that is formed in the reaction was measured at A_{max} 750 nm.

2.6 SDS-PAGE analysis

To analyze the molecular weight of the protein chains in the collagen dispersions and to assess the presence of different collagen types or collagen breakdown products sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. The method was adopted from Faraj *et al.* (2011). Briefly, 2.5 mg of lyophilized collagen was suspended in 150 μl 2x concentrated sample buffer (4% SDS, 20% (v/v) glycerol, 0.125M TRIS/ HCl pH 6.8, 20% (v/v) 2-β mercaptho-ethanol, bromo phenol blue) and heated for 10 min at 70°C. Both samples, and controls were loaded on an 8% (w/v) polyacrylamide gel (0.37M TRIS, final concentration is 0.1% SDS for both running and stacking gel; running buffer: TRIS/ glycine 2.5mM/ 0.2M). Running the gel took 1.5 hours at 100V until

the bromo phenol blue front was at the end of the gel. Staining was done with 0.1% (w/v) Coomassie Brilliant Blue solution (50% (v/v) methanol and 10% (v/v) acetic acid; R-250, VWR International BV, Amsterdam**,** The Netherlands) overnight**.** Destaining was performed using a solution containing 50% (v/v) methanol (Macron Fine Chemicals, VWR International BV, Amsterdam**,** The Netherlands) and 10% (v/v) acetic acid (Boom-lab, Meppel, The Netherlands) until all bands were clearly visible. Each dispersion (n=3) was analysed on the gel.

2.7 Rheology

Flow behavior can give an indication of the applicability of the dispersions for use in a co-extrusion system. The flow behavior of the different dispersions was measured with an oscillatory rheometer type AR2000 (TA Instruments, New Castle, USA) equipped with a Peltier plate and a water bath. The rheology measurements were performed based on Oechsle *et al.* (2016), with slight modifications. Oscillatory measurements, using a 40 mm diameter (hard-anodised aluminium) plate-plate geometry were used to analyse the collagen dispersions. To determine the linear viscoelastic range, stress sweeps were performed at 1Hz by applying oscillatory stress from 0.01 to 1000Pa at 5°C, followed by frequency sweeps performed in the linear viscoelastic range applying 0.1% strain from 1 to 100 rad/s. The complex viscosity η^* was determined as a function of angular frequency ω by applying Eq. 1 (Macosko, 1994):

$$\left[\left(\frac{G''}{\omega}\right)^2 + \left(\frac{G'}{\omega}\right)^2\right]^{1/2} \quad \text{(Eq. 1)}$$

To calculate the dynamic consistency index *k** and the dynamic power law factor *n** by applying Eq. 2 (Keogh and O'Kennedy, 1998), the complex viscosity η^* as a function of the angular frequency ω was plotted.

$$\eta^* = k^* \omega^{n^* - 1} \quad \text{(Eq. 2)}$$

By applying 0.1% strain from 5 to 60°C with ramp rate of 2°C/ min at 1Hz temperature sweeps of the dispersions were performed. The temperature at which G' (Pa) started to decrease as a measure of the helix to random coil (i.e., gelatin) transition was determined. The three replicates of each dispersion were measured in duplicate, whereby the average of the duplicates was used in the statistical analysis.

2.8 Determination of primary amine group content

Using 2,4,6-trinitrobenzenesulfonic acid (**TNBS**) the number of primary amine groups present in the lyophilized chicken dispersions was determined. The method was adopted from Buttafoco *et al.* (2006). An aqueous solution of 4% w/v $NaHCO_3$ (1 ml) was used to incubate the lyophilized samples of 1 mg for 30 min, followed by the addition of

a solution of 0.5% w/v TNBS (1 ml) in MilliQ water and incubation at 40°C for 2 h. HCl (3 ml, 6 M) was added followed by hydrolyzing the samples at 60°C for 90 min. The mixture was diluted in a 96 well plate 1:1 with MilliQ water. The absorbance was measured at 420 nm, using a spectrophotometer (Bio-Tek, Bad Friedrichshall, Germany). A glycine calibration curve was used to determine the concentration of amine groups. The three replicates of the dispersions were measured in triplicate, whereby the average value was used in the statistical analysis.

2.9 Film preparation

To evaluate the dispersions for film forming ability and strength, films were prepared by placing 5 g of each dispersion on a stainless-steel board, between two layers of plastic sheets, and flattening them with a stainless-steel roller. The roller had a recess of 0.5 mm to achieve uniform film thickness. Sodium chloride solution (24%) was added by a Pasteur pipette to the side of the collagen film between the two plastic sheets, and by carefully lifting the plastic sheet, to the top of the collagen film, the collagen precipitated. Ten films were made (dimension 150 mm x 40 mm) from each of the three replicates (per skin type) and evaluated for their mechanical and thermal properties.

2.10 Thermal transition measurement

Differential Scanning Calorimetry (**DSC**) measurements were performed to study the thermal behavior of the chicken skin dispersions and the precipitated films. The endothermal transition of the dispersions was measured by a DSC (Q1000, TA Instruments, New Castle, DE, USA) equipped with a cooling unit (RCS40). About eight mg of aqueous collagen dispersion or precipitated film were hermetically sealed in a T_{zero} aluminum pan. The sample was equilibrated at 1°C for 5 min. For the collagen dispersion, a temperature ramp was performed from 1 to 60°C, at 5°C/ min, while the precipitated collagen films were scanned at a temperature ramp of 1 to 80°C, at 5°C/ min. For all measurements an empty aluminum pan was used as the reference probe. Values determined were: temperature at which denaturation of the collagen helix started (T_{onset}), temperature at which 50% of the collagen helix has unfolded (T_{peak}), and the enthalpy value of denaturation (ΔH), using the DSC software (Universal Analysis 2000 (Version 4.5A), TA instruments). The three replicates of each skin type were measured in duplicate, whereby the average of the duplicates was used in the statistical analysis.

2.11 Mechanical properties

Strength and elasticity parameters of the films, cut into 150 mm x 40 mm strips and precipitated with 24% NaCl solution, were determined by using a texture analyzer (TA. XT2, Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell. The tensile measurements were performed by fixing the film with tensile grips (A/TG), employing a crosshead speed of 40 mm/s, gripper initial distance at 30 mm, target distance at 100

mm, and a break sensitivity at 1.00 N. The thickness (0.5 mm) and width (40 mm) of the films were used for the tensile stress calculations. Tensile strength (maximum stress the film can handle prior to breaking) and the stiffness of the casing (maximum elongation the film reached just prior to breaking) were determined from the generated stress-strain curves. Ten films per batch were evaluated, whereby the average value was used in the statistical analysis.

2.12 Statistical analysis

Minitab Version 19 (Minitab Ltd., Coventry, UK) was used for the statistical analysis on all data. Data were checked for normality in both means and residuals. Non-normal distributed data of protein content was transformed (square-root) before analysis. A general linear model was used for analysis of variance (ANOVA) for the dry matter and protein content, thermal, rheological, and mechanical properties, and determination of primary amine group content. The heat flow data of the collagen films required a log transformation (LN) to get normal distributed data. The model used for dry matter and protein content, thermal and rheological properties and primary amine group content was:

$$Y = \mu + Type + Weight + Type*Weight + e$$

We also attempted to look at the age effect. However, it did not work with the original model, due to the small number of birds. Therefore, the following model was also evaluated:

$$Y = \mu + Type + Age + e$$

The model for mechanical properties was:

$$Y = \mu + Type + Weight + Type*Weight + Protein + e$$

And also the following model was applied:

$$Y= \mu + Type + Age + Protein + e$$

Where Y = dependent variable; μ = overall mean; Type = fast growing or slow growing broiler (FBC or SBC); Weight = 1.6 kg or 2.2 kg; Age = 28, 37 or 50 days; Interaction = 2-way interaction between Type and Weight; Protein = protein content of the dispersion added as a covariate; e = residual error. Data are presented as means ± S.D. Means were compared, after correction for multiple comparisons, using the Bonferroni post-hoc test ($p<0.05$).

3. Results and discussion

3.1 Evaluation of chicken collagen dispersions

3.1.1. Dry matter and protein content of chicken collagen dispersions

Dispersion prepared from FBC_2.2 kg contained the highest dry matter content of 14.6%, followed by SBC_2.2 kg, SBC_1.6 kg and SBC_1.6 kg, containing 12.3%; 12.2% and 12.2%, respectively. No significant interaction was found between broiler strain and weight and no significant differences in dry matter content was found for broiler strain or weight ($P>0.05$). A significant difference in dry matter was found between the broiler strains when the factor age was considered in the model ($P<0.05$), resulting in a significant higher dry matter content for FBC than for SBC. Dispersions prepared from SBC_1.6 kg contained the highest protein concentration of 74 ±3 µg/ mg followed by the dispersion prepared from FBC_1.6 kg, SBC_2.2 kg, and FBC_2.2 kg with 66±8; 66±6 and 52±6 µg/ mg, respectively. Protein concentration in the dispersions did not show an interaction between chicken type and weight ($P=0.507$) but was higher for the SBC than for the FBC chickens (69.39 vs 58.89; $P=0.011$) and was also higher for the 1.6 kg chickens than for the 2.2 kg (70.04 vs 58.23; $P=0.003$) and was higher for the 28 days old chickens than for the 37- or 50-days old chickens (77.58 vs 62.50 vs 53.96; $P=0.009$). Higher protein content for the SBC dispersions was expected as these chickens had more time to reach full growth compared to FBC chickens. This was also expected for the 2.2 kg chickens and for the 50 days old chickens; however, this is not reflected in the results. The values are inconsistent with the previous study by Suurs *et al.* (2022), where FBC skins at 42 days showed higher protein content (76.4 µg/ mg) compared to SBC skins at 56 days (73.5 µg/ mg). Differences between the two studies may be explained by dietary differences, age at processing, and housing. The chicken skins in the current study were obtained from a study described by Van der Eijk *et al.* (2022), where the chickens were raised in The Netherlands, under controlled conditions, while monitoring feed type and intake. The skins from the study by Suurs *et al.* (2022) were obtained from a local processing plant in Germany. These chickens were raised under commercial conditions with other feed type. Moreover, the chicken skins in the current study were processed at 37 days and 50 days for FBC and SBC, respectively, whereas in the study of Suurs *et al.* (2022), the chickens were processed at 42 and 56 days for FBC and SBC, respectively.

3.1.2 Soluble collagen content

Collagen dispersions used for the co-extrusion process are a mixture of fibrous and acid soluble collagenous material (Barbut *et al.*, 2020). Soluble collagen can be obtained during the chemical liming treatment (hydrolysis). In the current study, the soluble collagen

content was determined to obtain an indication of the dispersion composition regarding soluble and insoluble (fibrous) collagen content. Unfortunately, due to the limited amount of skin material available, it was only possible to analyze this for the SBC_2.2 kg and FBC_2.2 kg. For the SBC_2.2 kg, soluble collagen extracted from the skin was 45.8%. For the FBC_2.2 kg, it was 51.4%. In future research, it would be good to investigate the ratio of soluble versus insoluble collagen in the dispersion. Especially because in the co-extrusion process, a counter rotating nozzle is used to weave the fibrous collagen material in a crisscross direction to give the casing strength. Particularly, the insoluble collagen fibers in the film are oriented circumferential (Hoogenkamp *et al.*, 2015). So, a greater proportion of soluble collagenous material would result in casings with lower strength as this material cannot be aligned by the nozzle. However, a certain amount of soluble collagen is necessary as it provides a glue to the dispersion to hold the fibers and fibrils together.

3.1.3 SDS-PAGE

The presence of two distinct bands at approximately: 130 kDa and 150 kDa were shown at the SDS-PAGE gel (Fig. 2). These bands indicate α1(I)- and α2(I)-chains of type I collagen (Gojkovic *et al.*, 2014), and a β-chain. The β-chains consists of two α-chains. These findings correspond with data from Munasinghe *et al.* (2021), who extracted type I collagen from chicken skin and obtained α1 and α2 chains with 148 kDa and 130 kDa molecular weights, respectively. The SDS-PAGE showed no bands visible at the lower molecular weight, indicating the absence of degraded collagen.

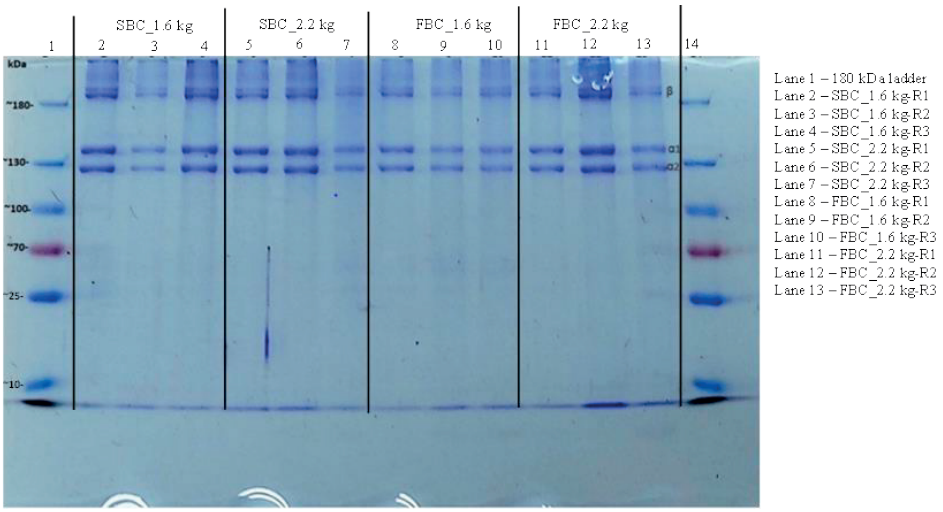

Figure 2. SDS-PAGE gel of the marker (lanes 1 and 14) and of the dispersions of slow growing broiler chicken of 1.6kg (SBC_1.6kg) (lanes 2 to 4); slow growing broiler chicken of 2.2kg (SBC_2.2kg) (lanes 5 to 7); fast growing broiler chicken of 1.6kg (FBC_1.6kg) (lanes 8 to 10); and fast- growing broiler chicken of 2.2kg (FBC_2.2kg) (lanes 11 to 13). Indicating the presence of collagen β, α1 and α2 collagen bands. R1, R2 and R3 represent the three replicates of each chicken type and weight.

For co-extrusion, it is important to have fibrous collagen material, i.e., insoluble fibrils and fibers, present in the dispersion as they can (i.e., with the counter rotating nozzle and the salt treatment) form a stable network that enables the film to shrink and stretch during cooking, as the meat batter contracts and expands during heat processing (Osburn, 2002). Bovine skin, the currently used raw material for casing production, contains type I collagen fibrils and fibers (Oechsle *et al.*, 2016). This study indicates that chicken skin collagen obtained by the current procedure contains collagen polypeptide chains, which in combination with the counter rotating nozzle action (alignment of fibrils and fibers), should be able to provide a stable collagen fibrils/ fibers network formation.

3.1.4 Rheology

3.1.4.1 Temperature profiles

The temperature profiles showed that at approximately 40°C all dispersions started to display a rapid decrease in elasticity (Fig. 3). This confirms the range of onset temperatures measured by DSC (Table 1). Broiler strain, weight and age had no influence on the temperature stability of the collagen dispersions ($P>0.05$). This temperature range is comparable to Suurs *et al.* (2022), where skin collagen of fast and slow growing broilers was investigated. For bovine collagen dispersions a rapid decrease in elasticity was observed between 30 and 45°C (Barbut et al, 2020). According to Paul and Bailey (2003) and Covington (2011), the variation in denaturation temperatures is correlated with the proportion of hydroxyproline in the different species. The higher the proportion of hydroxyproline, the higher the denaturation temperature. Hydroxyproline levels of the different broiler strains and weights were not measured. The interest is in comparing the chicken skin collagen with bovine skin collagen as the latter is currently used as the collagen source for dispersions for co-extrusion. Based on the study of Nik *et al.* (2014), chicken skin collagen would have higher denaturation temperature with hydroxyproline content of 121 residues/1000 compared to porcine and bovine skin containing 91 and 83 residues/ 1000, respectively. However, this is not reflected in the results when comparing the temperatures found by Barbut *et al.* (2020).

In the sausage production process, a heating step is used to cook the sausages (Suurs and Barbut, 2020). Hereby it must be prevented that the collagen helix unfolds into random coil structures (i.e., transformation into gelatin), since then the casing can feel sticky resulting in poor quality sausages as the casing will stick to the baskets (Suurs *et al.*, 2022). This means that collagen dispersions need a high denaturation temperature to prevent formation of random coil structures. Based on the results of the current study, all collagen dispersions appear to be suitable to be used in the co-extrusion application as their thermal stability is in the temperature range reported for bovine collagen dispersions (Barbut *et al.* 2020).

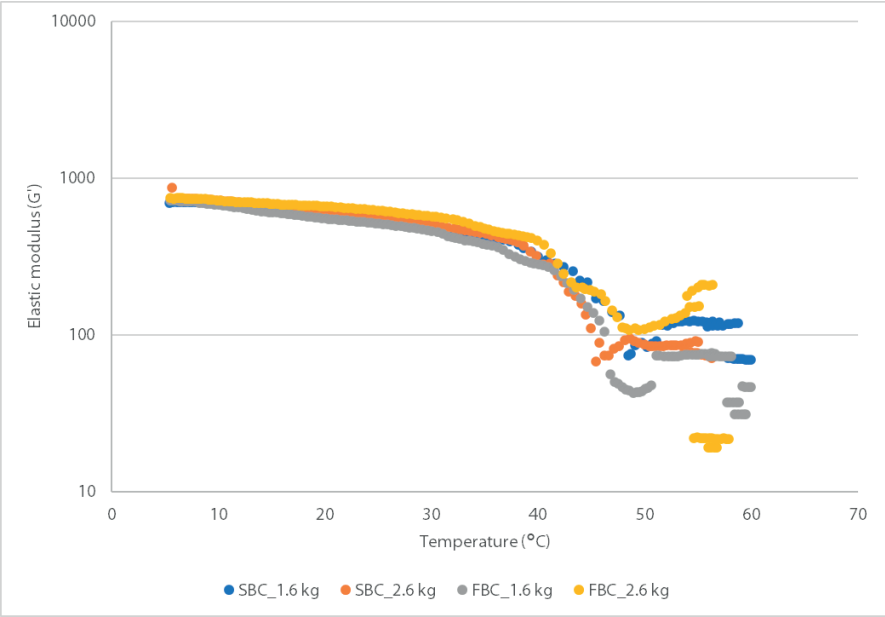

Figure 3. Elastic modulus (scans 5 to 60°C at 2°C/ min) of four chicken collagen dispersions: slow growing broiler chicken of 1.6kg (SBC_1.6kg); slow growing broiler chicken of 2.2kg (SBC_2.2kg); fast growing broiler chicken of 1.6kg (FBC_1.6kg); and fast growing broiler chicken of 2.2kg (FBC_2.2kg), all dispersions show a decrease in elasticity at 40°C (n=3 per treatment).

3.1.4.2. Complex viscosity

Flow properties of the dispersion provides valuable information for sausage producers using a co-extrusion system. Figure 4, where the complex viscosity η^* is plotted as a function of angular frequency (ω), shows that all dispersions exhibited a shear-thinning behavior, following a power-law model. Overall, the complex viscosity of the dispersions was comparable to the values reported by Suurs *et al.* (2022).

Figures 5a shows the dynamic consistency index (k^*) for the chicken collagen dispersions. No significant interaction was found between broiler strain and weight (both $P>0.05$) and also no significant differences were found for broiler strain, weight or age ($P>0.05$). The values measured in the current study are lower than the ones reported by Suurs *et al.* (2022), where values of 41 Pa s^{n^*} for FBC and 44 Pa s^{n^*} for SBC were recorded. Moreover, the values were much lower compared to the study of Oechsle *et al.* (2016), who reported a value of 606 Pa s^{n^*} for chicken skin collagen. The collagen extraction processes and dispersions' protein content probably resulted in large differences compared to current findings. Compared to Suurs *et al.* (2022), the acid swelling step in the current study was extended from 24 h to 48 h. This resulted in more swelling of the collagen and therefore probably a lower consistency value. Moreover, in the previous study,

there were clearly unswollen parts of collagen visible, while this was not the case in the current study. The skin collagen dispersion made by Oechsle *et al.* (2016) was prepared according to Bueker *et al.* (2009), where the dispersion was made by coarsly mincing the acid swollen hide pieces and then forcing it through a plurality of perforated disks arranged in series where the diameter of the individual holes becomes smaller from disk to disk. Finally, the collagen paste is tranferred to large kneaders. So, here they used a different way of producing the chicken collagen dispersion compared to the current study.

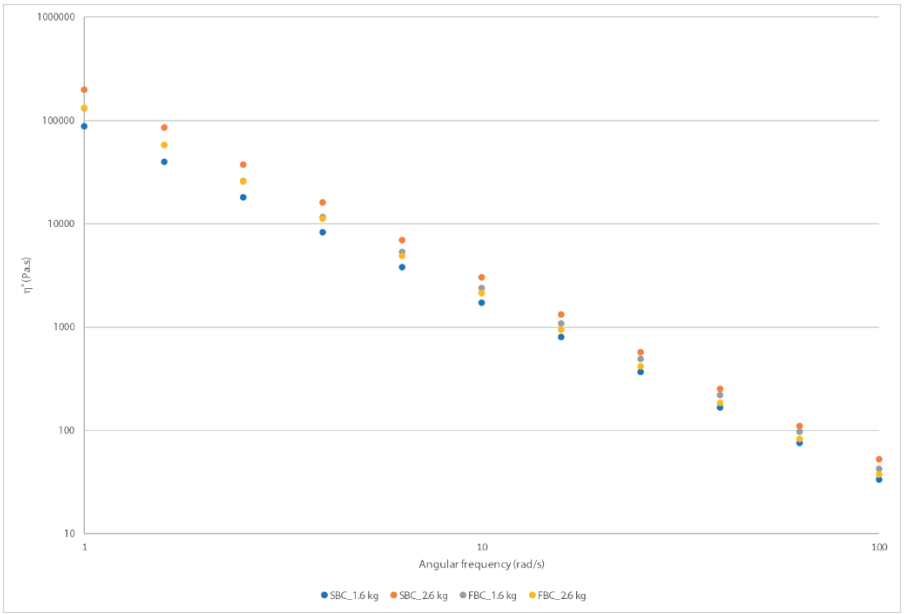

Figure 4. Complex viscosity η^* calculated from $\left[\left(\frac{G''}{\omega}\right)^2+\left(\frac{G'}{\omega}\right)^2\right]^{1/2}$ of chicken collagen dispersions of slow growing broiler chicken of 1.6kg (SBC_1.6kg), slow growing broiler chicken of 2.2kg (SBC_2.2kg), fast growing broiler chicken of 1.6kg (FBC_1.6kg), fast growing broiler chicken of 2.2kg (FBC_2.2kg) as a function of the angular frequency ω (n=3 per treatment), showing shear thinning behavior following a power-law model for all dispersions.

Shear-thinning ($n^* < 1$), shear-thickening ($n^* > 1$) or Newtonian ($n^* = 1$) behaviour can be indicated by the dynamic power law factor n^*. The dynamic power law factor n^* indicates shear-thinning ($n^* < 1$), shear-thickening ($n^* > 1$) or Newtonian ($n^* = 1$) behaviour (Keogh and O'Kennedy, 1998). Figure 5b shows that n^* values for all four dispersions were comparable and almost 0, which means they have almost only elastic behavior. The difference with Suurs *et al.* (2022) is that the current dispersions also contained small number of viscous components ($n^* = 0.03$), and especially the collagen from the lighter chickens. This is probably due to the younger age of the chickens, where less intra and

intermolecular crosslinks are formed and therefore more viscous components could be expected in the dispersion. The viscous components in the dispersion most probably originates from the soluble collagen. Unfortunately, no correlations between the n-value and soluble collagen content could be made, because there was not enough material available to measure the soluble collagen content. Comparable to the k^* and n^* values of two most used commercial bovine collagen dispersions (both dispersions measured: $k^* = 55$ Pa s^{n^*} and $n^* = 0.01$), the values for k^* from the current study are much lower. This could mean that the extrusion of the dispersions would not proceed satisfactorily, i.e., too low viscosity resulting in poor film forming capacity.

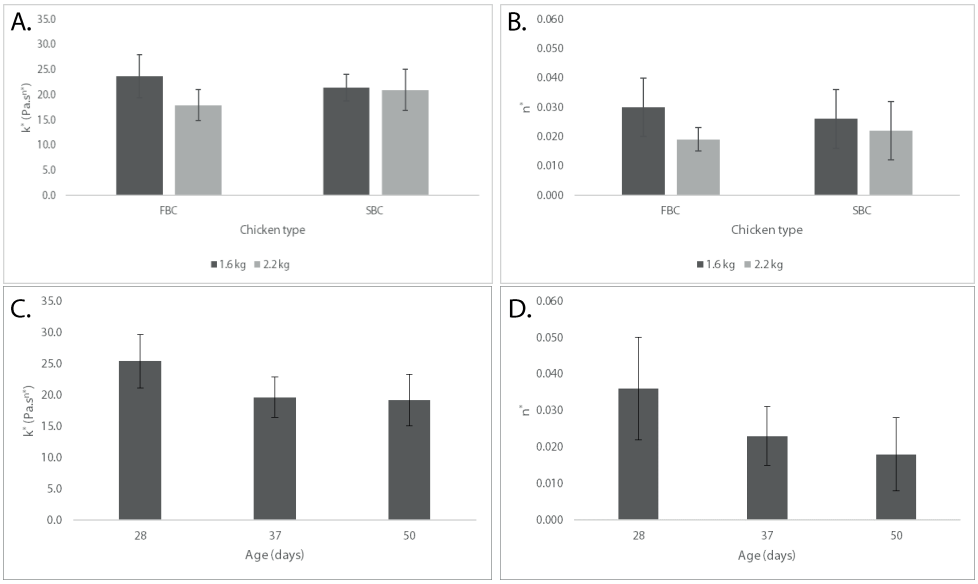

Figure 5. A and C. Dynamic consistency index (k^*), B and D. Dynamic power law factor (n^*) of collagen dispersions made from FBC (fast growing broiler chicken), and SBC (slow growing broiler chicken) weighing 1.6 and 2.2 kg. Bars represent mean values ± S.D.. A. Showing highest consistency value for FBC_1.6 kg, but no significant differences between the dispersions. B. Showing almost only elastic behavior for all dispersions. C. Showing highest consistency value for 28 days old chickens, D. Showing highest n^* value for 28 days old chickens.
A. P-value_strain = 0.88; P-value_weight = 0.23; P-value_strain*weight = 0.30.
B. P-value_strain = 0.71; P-value_weight = 0.11; P-value_strain*weight = 0.41.
C. P-value_age = 0.21.
D. P-value_age = 0.27.

3.1.5 Evaluation of the number of amine groups in chicken collagen dispersions

The number of crosslinkable groups was determined by assaying the free amine groups in the dispersions using the TNBS assay. The more free amine groups, the stronger the casing will be when adding a crosslinker that needs an amine group to react with; i.e.

for sausage production liquid smoke is used. Dispersion prepared from SBC_1.6 kg contained the highest number of amine groups 199 ± 20 nmol/ mg, followed by SBC_2.6 kg, FBC_1.6 kg and FBC_2.6 kg, containing 193 ± 28 nmol/ mg, 185 ± 20 nmol/ mg and 175 ± 25 nmol/ mg, respectively (Figure 6).

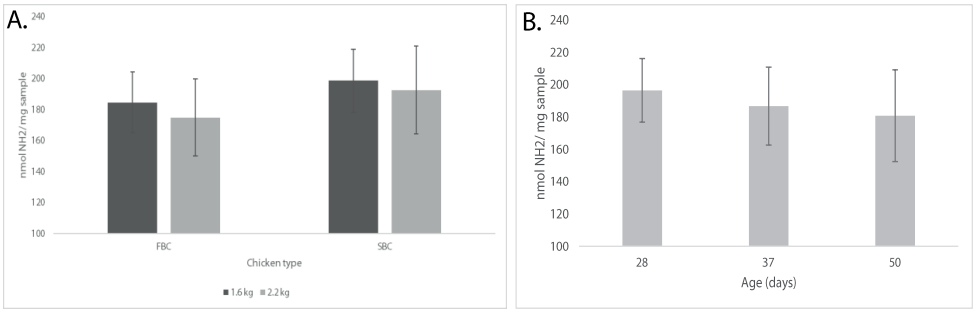

Figure 6 A. The number of NH_2 groups present in chicken collagen dispersions prepared from skins of slower growing broiler chicken of 1.6kg (SBC_1.6kg), slower growing broiler chicken of 2.2kg (SBC_2.2kg), fast growing broiler chicken of 1.6kg (FBC_1.6kg), and fast-growing broiler chicken of 2.2kg (FBC_2.2kg), showing no significant differences in free amine groups between the dispersions; B. The number of NH_2 groups present in chicken collagen dispersions prepared from skins of 28, 37 and 50 days old chickens, showing no significant difference in free amine groups between the ages. n=3 per treatment. Bars represent means ± S.D.
A. P-value_strain = 0.28; P-value_weight = 0.58; P-value_strain*weight = 0.89.
B. P-value_age = 0.84

No significant interaction was found between broiler strain and weight and no significant differences in free amine groups were found for broiler strain or weight (P>0.05). A lack of significant difference was expected for FBC_2.2 kg and SBC_1.6 kg as they were the same age at processing. The age difference between FBC_1.6 kg and FBC_2.2 and SBC_1.6 kg was probably too small to show changes in crosslinking. If a difference was to be expected, it would be for FBC_1.6 kg versus SBC_2.2 kg, as the SBC_2.2 kg chickens were actually older [28 days for FBC_1.6 kg (197 nmol/ mg) versus 50 days for SBC_2.2 kg (181 nmol/ mg)]. However, no significant difference was found for broiler strain or age (P>0.05). According to a study by Pines *et al.* (1996) no mature stable crosslinks could be detected in chicken breast skin up to 49 days of age.

The amine group content of three commercial bovine collagen dispersions was determined on 284 ±12 nmol/ mg, 442 ± 152 nmol/ mg, and 378 ± 56 nmol/ mg (unpublished data). The results indicate that there can be a considerable spread in amine group content between different commercial bovine collagen dispersions. Moreover, compared to the chicken collagen dispersions the number of amine groups of the bovine collagen dispersions is higher. The differences among the bovine collagen dispersions and with

the chicken collagen dispersions may have to do with the difference in production method, i.e., time in the alkaline solution, whereby deamidation by hydrolysis of the sidechains of glutamine and asparagine residues, the number of amine groups can be reduced as NH_2 groups will be split off and NH_3 is formed (Suurs & Barbut, 2020).

Considering that broiler strain, weight and age had no significant effect on the number of cross-linkable groups, all chicken skin sources in this study appears to be acceptable for casing production and the corresponding co-extrusion technology. However, considering the lower amount of amine groups in the chicken collagen dispersions, the casings would be less strong after crosslinking with liquid smoke compared to bovine collagen dispersions.

3.1.6 Differential Scanning Calorimetry (DSC) of collagen dispersions

DSC measurements were carried out to investigate the collagen transformation temperatures (Suurs *et al.*, 2022). The essence of collagen denaturation is the removal of hydrogen bonds. Denaturation of collagen consists of the disruption of the secondary and tertiary collagen structures. The collagen denaturation temperature increases with the age of the animal due to a higher degree of lysine-hydroxylysine covalent crosslinks formation between the collagen molecules (Jolanta & Piotr, 2010; Noorzai & Verbeek, 2020). As a result of denaturation, the collagen helix is transformed into a coil (Jolanta & Piotr, 2010). In the current study, no significant interaction was found between broiler strain and weight ($P>0.05$) on T_{onset}, T_{peak} and enthalpy. Furthermore, no main effects of broiler strain, weight and age were found (Table 1), suggesting that broiler strain, weight and age have no influence on the thermal properties of the prepared chicken skin dispersions. This was expected as the age difference between the chickens investigated was rather small.

The enthalpy corresponds with the number of hydrogen bonds in the triple helix that provide stability to the helix (Schroepfer and Meyer, 2017). Paul and Bailey (2003) mentioned that by measuring the enthalpy, an indication of the integrity of the collagen molecule can be provided. Degradation of the collagen results in lower enthalpy, whilst more crosslinking increases the denaturation temperature, resulting in higher enthalpy values. The current study indicates that the collagen dispersions prepared from the skins of heavier birds had higher integrity compared to the lighter birds, as the enthalpy increases with increasing weight of the birds (Table 1), which in turn may be related to age of the chickens. The data showed an increasing trend for enthalpy value with increasing age of the chickens (28 days 0.49 J/g vs 37 days 0.53 J/ g vs 50 days 0.61 J/g). However, no significant difference was found between the age of the chickens and the enthalpy ($P>0.05$).

Table 1. Endothermic peaks analysis from the differential scanning calorimeter (DSC) thermograms. Each broiler type and weight [fast growing broiler chicken of 1.6kg (FBC_1.6kg); slow growing broiler chicken of 1.6kg (SBC_1.6kg); fast growing broiler chicken of 2.2kg (FBC_2.2kg); slow growing broiler chicken of 2.2kg (SBC_2.2kg)] were determined in duplicate as collagen dispersion and dehydrated films (n=3 per treatment). Means ± standard deviations.

Type	Dispersion/ Film	T_{onset} (°C)	T_{peak} (°C)	Enthalpy ΔH (J/g)
FBC_1.6kg	Dispersion	39.06 ± 1.07	41.54 ± 1.46	0.28 ± 0.09
SBC_1.6kg	Dispersion	39.11 ± 1.13	41.77 ± 1.31	0.22 ± 0.06
FBC_2.2kg	Dispersion	38.78 ± 0.34	41.33 ± 0.56	0.40 ± 0.33
SBC_2.2kg	Dispersion	38.65 ± 0.40	41.69 ± 0.63	0.38 ± 0.11
P-value_strain		0.72	0.55	0.77
P-value_weight		0.62	0.70	0.38
P-value_ strain*weight		0.49	0.90	0.74
P-value_age		0.69	0.92	0.63
FBC_1.6kg	Film	55.87 ± 4.86	60.25 ± 5.54	0.23 ± 0.07
SBC_1.6kg	Film	54.67 ± 5.88	59.22 ± 7.04	0.31 ± 0.13
FBC_2.2kg	Film	50.07 ± 4.03	54.15 ± 5.08	0.28 ± 0.06
SBC_2.2kg	Film	54.32 ± 4.74	58.88 ± 4.35	0.41 ± 0.16
P-value_strain		0.43	0.38	0.52
P-value_weight		0.13	0.14	0.54
P-value_ strain*weight		0.25	0.25	0.98
P-value_age		0.16	0.16	0.83

3.2 Evaluation of chicken collagen films

3.2.1 Differential Scanning Calorimetry (DSC) of collagen films

Comparable to Suurs *et al.* (2022), the chicken collagen dispersions were made into a film by partial dehydration (using 24% NaCl as done in the industry during the co-extrusion process), and this resulted in a higher thermal stability for all dispersions (Table 1). No significant differences in T_{onset}, T_{peak} and enthalpy were found between the different collagen films (Table 1), indicating that broiler strain, weight and age have no influence on the thermal properties of the films. Barbut *et al.* (2020) and Suurs *et al.* (2022) also showed a significant increase in thermal stability of chicken and bovine collagen dispersions that were transformed into collagen films by salt (24%) precipitation. A compact structure of the fibers is formed due to the decreased moisture content as the salt induces dehydration. McPherson *et al.* (1986) also mentioned an increased denaturation temperature resulted in a stronger association of bovine collagen fiber structure. So,

from the perspective of the collagen films there is no preference which chicken dispersion should be used for casing production and corresponding co-extrusion technology.

3.2.2 Mechanical properties of collagen films

The ability to form a strong film (from the dispersions) is a very important aspect to make sausage casings in the co-extrusion process. First, the films must be strong enough to withstand the pressure of the meat dough during the partial drying and cooking. Second, the sensory perception of "bite" is determined by the strength of the casing together with the ground meat. No significant interaction was found between broiler strain and weight. Figure 7a and 7b demonstrate that there were also no significant main effects of broiler strain and weight (both $P>0.05$) in the tensile strength and Young's modulus when films prepared from the different dispersions were exposed to 24% NaCl solution. Moreover, the age of the chickens had no effect on the tensile strength or Young's modulus ($P>0.05$). However, the results indicate that in general the slow growing chickens showed lower tensile strength and stiffness values compared to the fast growing chickens. Moreover, the results also indicate that the tensile strength of films prepared from the skins of lighter chickens were less compared to the heavier chickens. The differences in texture are not originating from differences in protein content between the dispersions as no significant difference was observed ($p>0.05$) when protein content was added as a co-variate to the statistical models. At this point, no verification can be done to check whether the differences in tensile strength and stiffness values can be attributed to the difference in the proportion of soluble collagen, as there was not enough material to evaluate the amount of soluble collagen of the lighter chickens (FBC_1.6kg and SBC_1.6kg).

The co-extruded films containing 4% chicken skin collagen (normalized to protein content) from Oechsle *et al.* (2017) showed tensile strength values of 0.07 MPa, which are comparable to the values found in our study. Compared to the values measured for bovine collagen dispersions by Barbut *et al.* (2020), the values for tensile strength found in this study are about 5 times lower. This has probably to do with the orgin (bovine versus chicken collagen) and age of the raw materials when processed. Steers are processed at 18 – 24 months, whereas the chickens in the current study were 28 to 50 days old. As the collagen fibril diameter increases with age (Miller, 1983), stronger films should be produced from dispersions prepared from older animals' skin. Probably, there is an optimum in the animal's age, because at a certain point (depending on the origin) the collagen fibers in the skin are crosslinked and the films prepared from dispersions with these fibers will exhibit lower mechanical strength (Suurs *et al.*, 2022). Moreover, crosslinked fibers show more resistance to swelling and have a lower water-holding capacity (Miller, 1983), which strongly influences the film forming ability and tensile properties. Considering that broiler strain and weight had no influence on mechani-

cal properties of the films, all chicken skin sources evaluated in this study appears to be acceptable for casing production and the corresponding co-extrusion technology. However, sausage manufacturers must consider getting a less tough casing when using chicken skin collagen than when working with bovine skin collagen.

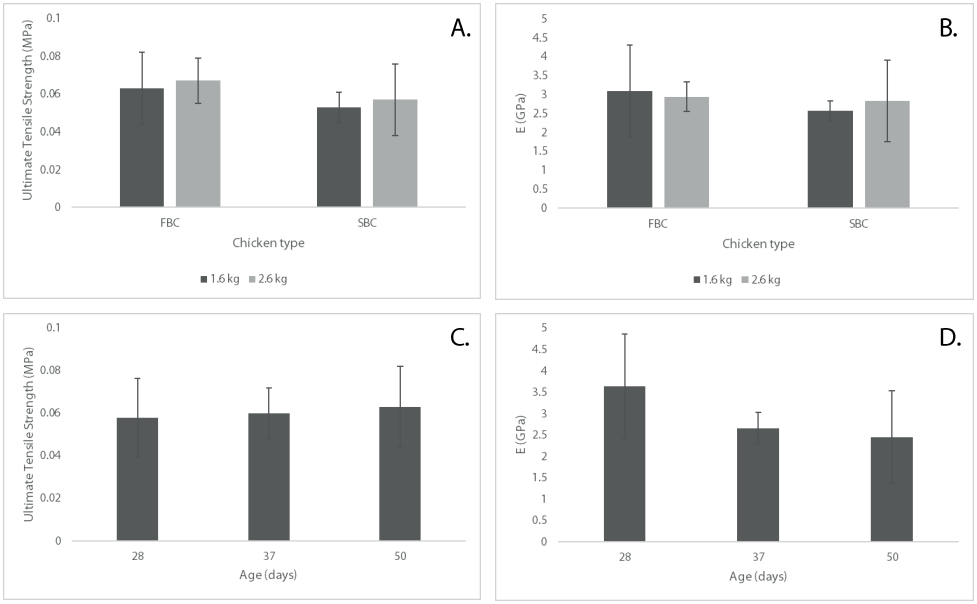

Figure 7. Mechanical properties of collagen films (produced by exposing to 24% NaCl); A) Ultimate tensile strength (UTS) and B) Stiffness (E) for slow growing broiler chicken of 1.6kg (SBC_1.6kg), slow growing broiler chicken of 2.2kg (SBC_2.2kg), fast growing broiler chicken of 1.6kg (FBC_1.6kg), fast growing broiler chicken of 2.2kg (FBC_2.2kg); showing no significant differences in UTS and E values for the different films. C) Ultimate tensile strength (UTS) and D) Stiffness (E) for 28-, 37- and 50-days old chickens, showing no significant differences in UTS and E values. n = 3 per treatment. Bars represent means ± S.D.
A. P-value_strain = 0.29; P-value_weight=0.62; P-value=0.99.
B. P-value_strain = 0.54; P-value_weight = 0.92; P-value_strain*weight = 0.68
C. P-value_age = 0.98.
D. P-value_age = 0.65.

4. Conclusions

Based on the properties measured in the current study the chicken collagen dispersions prepared of skins from slow and fast-growing broilers have the potential of being a suitable source of collagen for the co-extrusion process. However, attention should be given to the rheological properties as the k^* values of the dispersions are rather low. Furthermore, the fact that the strength of the films prepared from the chicken collagen

dispersions is lower compared to the films prepared from bovine collagen dispersions, might have the consequence that the bite characteristics of these films will be different, and therefore must be considered when using this material.

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CHAPTER 4

Evaluation of cattle skin collagen for producing co-extrusion sausage casing

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Food Hydrocoll: 2023; 140: 108595

Abstract

Co-extrusion is a fully automated sausage production process that employs a continuous stream of collagen dispersion to encase the extruded meat mass to form an endless sausage rope, that is later crimped into links of selected sizes. Fibrous and soluble type I collagen dispersions obtained from bovine skins of animals aged 18 – 36 months is used as the raw material for these dispersions.

In this study, the chemical and physical properties of cattle skin collagen preparations from four sources [American Calf (AC), Dutch Heavy Veal (DHV), Danish Ox and/or Heifer (DOH), and Heavy German Cow (HGC)] were investigated for their potential application as collagen source for co-extrusion.

All dispersions exhibited shear-thinning behavior, following a power-law model with k^* values for HGC, DHV, AC, and DOH dispersions of 59, 68, 95 and 114 Pa s^{n^*}, respectively. Rheological measurements showed for all dispersions a decrease in elasticity and loss modulus at 35 - 40°C. SDS-PAGE indicated the presence of α1(I)- and α2(I)-chains of type I collagen for all dispersions. The mechanical strength of the films was 1.6, 1.6, 1.3 and 1.2 MPa for films prepared from AC, DOH, DHV and HGC dispersions, respectively. After crosslinking a 27% reduction of free amine groups was found for HGC and DOH, followed by 26 and 19% for AC and DHV, respectively.

Based on the properties of the dispersions and the films in relation to the co-extrusion process AC, DHV and DOH are potentially suitable as an alternative collagen source.

1. Introduction

For many centuries, consumers around the world have enjoyed traditional sausages made by stuffing meat into natural sheep/ hog casings (Wijnker, 2009). Today many sausages are still made this way and then hung on sticks, which are loaded onto carts, and placed in a smokehouse for further processing by drying/ smoking and heating. From an industry's perspective there are basically two alternatives to the smoking and cooking processes: 1) a semi-continuous operation, using sticks that are automatically loaded into the smokehouse; 2) co-extrusion technology in which a semi liquid material (i.e., collagen paste) is introduced onto an endless stream of meat dough (coming out of a meat stuffer), and the collagen is initially gelled in place by the application of a saturated salt solution (typically NaCl, that quickly withdraws water from the gel) and later the continuously filled casing (filled with the meat) is split into individual links/ sausages. The follow up steps of thermal drying and the use of liquid smoke (contains aldehydes that crosslink protein molecules) are used to 'strengthen/ cement' the casings structure. Unlike the traditional way of sausage making, co-extrusion eliminates the intermediate stages of preparing and storing pre-made casings as in natural or manufactured casings (Suurs and Barbut, 2020). The collagen dispersion used for co-extrusion is initially prepared the same way as the dispersion used for traditional fabricated casings (i.e., arrive to the plant as dried fully prepared to be stuffed casings). The difference lies in the fact that the co-extrusion dispersion (arrive to the plant as gel) is prepared from the same raw materials and goes through the initial processing stages (deliming the skins, washing, acidification and homogenization of the collagen fibers which yields a solid content of 4 -5%). At this point the dispersion is packed and sent to the meat plant, while the traditional fabricated casing is going through extrusion to create the casing shape (using a special tunnel), which is then dried and crosslinked (Sobanwa, 2021). To produce traditional manufactured collagen casing a coagulation bath of sodium or ammonium salt at a pH of 12 (to neutralize the acid and to promote collagen fiber shrinkage), a plasticizer such as glycerol and sorbitol and crosslinking agents such as glutaraldehyde or liquid smoke are used to reduce brittleness and improve casing strength. In the co-extrusion process, a sodium salt bath at neutral pH is used to precipitate the collagen dispersion into a collagen casing and no plasticizers are used. Furthermore, crosslinking with glutaraldehyde is not allowed in the co-extrusion process as it is not approved by the Food and Drug Administration (FDA) as an ingredient for food products. After crosslinking, the casings produced according to the traditional process are inflated with air and dried to a final moisture content of about 13 – 18%. The casings are ready to be stuffed with meat batter, whereas in the co-extrusion process the casing is being formed as the sausage is produced (Savic and Savic, 2016; Sobanwa, 2021).

The co-extrusion process is characterized by several steps, which are generally divided into five zones (Fig. 1), to obtain a cooked smoked sausage.

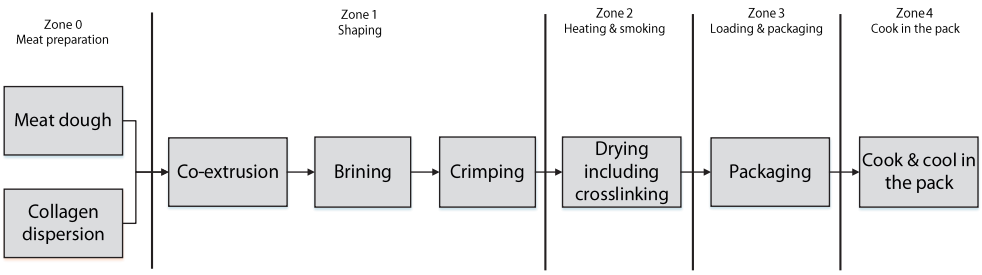


Figure 1. Schematic overview of the co-extrusion process for preparing cooked smoked sausages, indicating the different zones. Zone 0: meat preparation; Zone 1: shaping of the sausage; Zone 2: heating and smoking; Zone 3: automatic loading and packaging of the sausages; Zone 4: cook the sausages in the pack until T_{core} is 74°C and chill afterwards.

Zone 0 is related to the meat preparation step and receiving the raw collagen dispersion (as it is being delivered by the supplier). Zone 1 is related to shaping of the product, creating a casing as the sausage is being produced. Here a saturated salt solution is used to quickly remove water from the gel and help precipitate the collagen dispersion into a film. In Zone 2, the sausages enter the drying cabinet to further stabilize the casing by both air-drying and crosslinking with smoke condensates (Suurs and Barbut, 2020; Kobussen *et al.*, 2000). The aldehydes present in the smoke condensate are the most important ones for this process, as they are capable to induce covalent crosslinkages between collagen fibers and fibrils (Toledo, 2007). The covalent bonding strengthen the network structure thereby enhancing the mechanical properties of the collagen film (Morgan *et al.*, 1988; Bontjer *et al.*, 2011; Zhang *et al.*, 2022). In Zone 3, sausages are automatically loaded into packages. In Zone 4, packed sausages are cooked to an internal temperature of 74°C to obtain a safe product (Savic and Savic, 2016).

In every step of the process, the collagen paste must have certain properties to achieve a well-accepted sausage product. An important feature of the co-extrusion process is that a collagen paste is used, consisting of fibrous and soluble type I collagen with approximately 3-5% protein (Barbut *et al.*, 2020). The main source of collagen for co-extrusion is bovine skin/ hide, and more specifically the corium layer of animals aged 18 – 36 months, a by-product of the leather industry (Sobanwa, 2021). According to Sobanwa (2021) the hide's corium of animals at this age is more fibrous, stronger and contains less soluble collagen compared to corium of younger animals. However, different skin types are available at the leather tanneries, e.g., of veal calves, heifers and oxes. Their hide off-cuttings, which cannot be used by the leather industry (Noorzai *et al.*,

2019), usually end up in landfill. Extracting collagen from these off-cuttings can greatly increase their value (Noorzai *et al.*, 2019; Matinong *et al.*, 2022). However, as mentioned by Noorzai *et al.* (2019) and Matinong *et al.* (2022), the structure of a hide is influenced by age, sex, diet, and environment. In this study, we examined whether cattle skins, obtained from alternative sources, may be used to produce high-quality co-extrusion casings. The study compared the chemical composition of skins' collagen from two different veal calf breeds (used for meat production), a mixture from Danish ox and heifers (used for working [ox] and meat production [heifer]), and heavy German cows (used for meat/ milk production), employing both biochemical and physical measurements (note: a mixture of hides from oxen and heifers was used, because this was offered as such by tannery). The overall aim of the study was to evaluate potential sources of cattle collagen for the co-extrusion process.

2. Material and methods

2.1 Experimental design

Cattle skins from different types [(American Calf (**AC**), Dutch Heavy Veal (**DHV**), Danish Ox or Heifers (**DOH**) and Heavy German Cow (**HGC**)] were collected from a local tannery (ECCO, Dongen, The Netherlands). The main difference between DOH versus HGC is that cows are used for meat production and breeding purposes, whereas the oxen are especially used as working animals (Noorzai *et al.*, 2019). Furthermore, there is a difference in slaughter age between the different cattle species: calves 6 – 8 months, ox/ heifers at a maximum age of 30 months, and cows 36 – 60 months. One skin was available per animal species, the origin on the animal's body was unknown i.e., of the spine region or abdomen. The whole skin (including the hair/ coat) of each group was collected and divided into four portions to prepare four dispersions (n=4) per source. The four portions were stored at -18°C, until processing.

2.2 Preparation of skin collagen dispersions

A multistep process was used to obtain the dispersions prior to determining their physical and chemical properties (Fig. 2). The procedure was performed according to Noorzai *et al.* (2019) with slight modifications.

Briefly, skins were thawed and cut into pieces of approximately 200 g. Skins were incubated in 0.1 M NaOH (Boomlab, Meppel, The Netherlands) at a sample: solution ratio of 1:6 (w/v - based on the wet weight of the original sample, 1 g of sample with 6 ml of solution). The mixtures of AC and DHV were soaked for 24 h, and DOH and HGC were soaked for 48 h in NaOH at 4°C. Given the age difference between AC and DHV versus

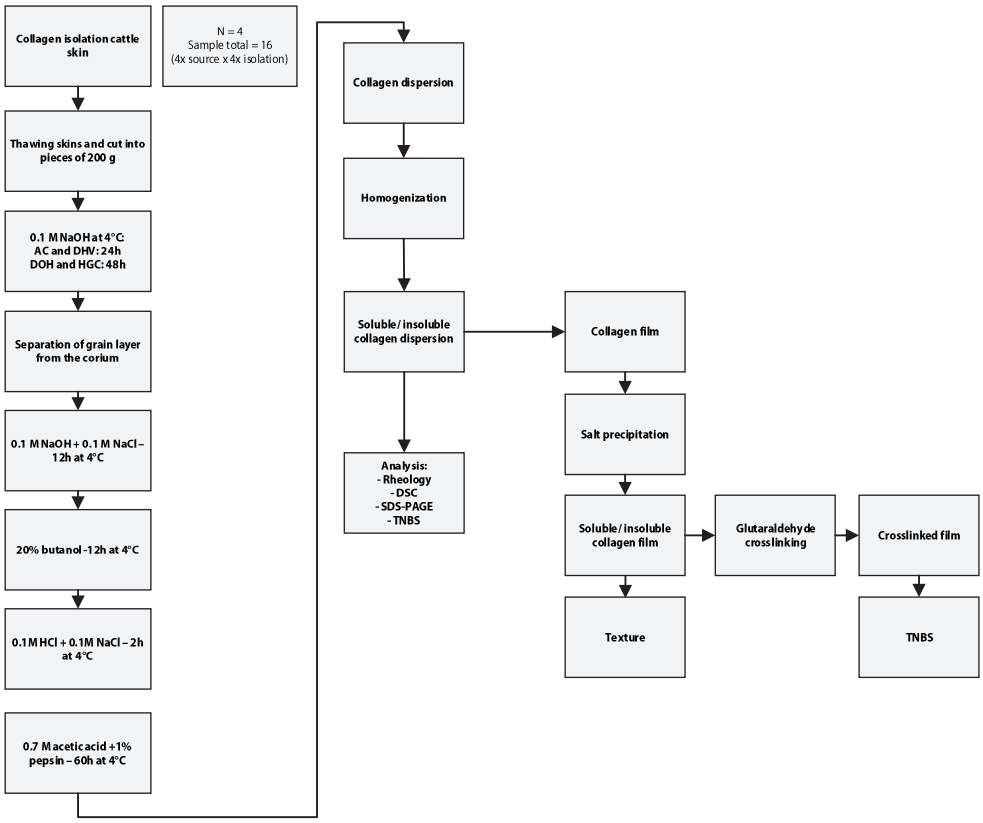


Figure 2. Flow chart of the extraction methods used to obtain collagen dispersions and films from the different cattle skins, including an overview of the analysis methods for both the dispersions and films. The abbreviations of the methods used are as follows: Differential scanning calorimetry (**DSC**), Sodium dodecyl sulfate polyacrylamide gel electrophoresis (**SDS-PAGE**), and 2,4,6-trinitrobenzenesulfonic acid (**TNBS:** amine group content).

DOH and HGC, more crosslinks are expected in the latter two and therefore the time in the NaOH was extended. Due to swelling of the skin, it was possible to separate the grain layer (including the coat) from the corium with a knife. NaOH was removed by washing with distilled water and filtering (2.5 mm mesh) until neutral pH was reached. Thereafter, fat was removed by adding first 0.1 M NaOH + 0.1 M NaCl (Boomlab) at a sample: solution ratio of 1:6 (w/v – based on the weight of the swollen skin pieces), whereby the solution was renewed every 2 h for at least 12 h, and then 20% butanol (Chemlab, Zedelgem, Belgium) at a sample: solution ratio of 1:6 (w/v – based on the weight of the skin pieces) was added. Mixtures were stored overnight at 4°C. Samples were later washed and filtered (2.5 mm mesh) with distilled water until the pH of the wash water reached neutral pH. After defatting, the skin pieces were cut into smaller pieces (1 x 1 cm) and soaked in 0.1 M HCl (Boomlab) + 0.1 M NaCl demineralization

solution with a sample: solution ratio of 1:6 (w/v) for 2 h at 4°C. The mixture was then filtered through a double layer cheese cloth, followed by rinsing with distilled water until the pH of the wash water reached neutral pH. Samples were swollen in 0.7 M acetic acid (Supelco, Zwijndrecht, The Netherlands) and 1% (w/w) pepsin (Merck, Darmstadt, Germany) for 60 h at 4°C at a sample: solution ratio of 1: 2 (w/v). The pepsin will only attack the non-triple helical domains of native collagen and leaves the helical portions intact (Zhang *et al.*, 2006; Matinong *et al.*, 2022). Acid not taken up by the collagen was filtered out (2.5 mm mesh), collected, and kept for mixing. Samples were pre-mixed in a food processor (Braun, type 3202, Kronberg im Taunus, Germany) with a sample: solution ratio of 2:1 (w/v, based on the weight of swollen skin pieces), followed by homogenization with a water-cooled high shear mixer (Marel, Boxmeer, The Netherlands) until a homogenous collagen dispersion was obtained.

2.3 Soluble collagen content of cattle skin collagen dispersions

Additional samples from the AC, DHV, DOH and HGC skins were prepared to obtain an indication of the amount of soluble collagen in each dispersion. Samples were obtained similarly, but after swelling in 0.7 M acetic acid plus 1% pepsin (w/w), the skin material was filtered (2.5 mm mesh), and the filtrate was collected and precipitated to obtain soluble collagen. NaCl crystals *(*Boomlab) were used for the precipitation until a final concentration of 2.6 M was reached. The solution was stirred and left for 24 h at 4°C. The precipitates were collected by centrifuging (Thermo Scientific, IEC, CL10, Fixed Angle Rotor F-G1, Waltham, Massachusetts, USA) at 3,700 g for 30 min. The pellet was dialyzed (Ø 17.5 mm, molecular weight cutoff at 14 kDa; Medicell, London, United Kingdom) in approximately 20 volumes of 0.7 M acetic acid for 24 h at 4°C.

2.4 SDS-PAGE analysis of collagen dispersions

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the molecular weight of the protein chains in the collagen dispersions (soluble part), and to assess the presence of collagen breakdown products. The method was obtained from Faraj *et al.* (2011). Briefly, 2.5 mg of lyophilized dispersions was suspended in 150 µl sample buffer followed by heating for 10 min at 70°C. An 8% (w/v) polyacrylamide gel was prepared to load both samples and controls. The gel was run at 100 V until the Bromophenol blue front was at the end of the gel. Gels were stained overnight with 0.1% (w/v) Coomassie Brilliant Blue solution (R-250, VWR International BV, Amsterdam**,** The Netherlands)**.** A solution containing 50% (v/v) methanol (Macron Fine Chemicals, VWR International BV) and 10% (v/v) acetic acid (Boomlab) was used for de-staining until bands were clearly visible. Each dispersion prepared from the different skin type (n=4) was analyzed on gel.

2.5 Moisture and protein content of collagen dispersions

Dry matter content was determined by lyophilization (LP-03, IlShin BioBase Europe, Ede, The Netherlands), where approximately 25 g of the collagen dispersion was lyophilized, after which the dry material was weighed and calculated. Four replicates of each skin type were measured once, whereby the average per skin type was used in the statistical analysis.

Protein was assessed according to Lowry *et al.* (1951). In short, 10 mg of lyophilized dispersions were incubated in 1 ml papain digestion buffer of 50 mM phosphate buffer at pH 6.5 containing 2 mM cysteine, 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 U papain (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 16 h at 65°C. After digestion, the samples were centrifuged (5 min; 13,000 g) and supernatants were used for protein measurements. A calibration curve was made, using bovine serum albumin (BSA) at concentrations of 0 to 250 ng/ml in papain digestion buffer. The color reaction that occurs has a characteristic blue color with A_{max} at 750 nm. Four replicates of each skin type were measured in triplicate, whereby the average of the triplicates was used in the statistical analysis.

2.6 Thermal transition measurement of cattle skin collagen dispersions

Differential Scanning Calorimetry (DSC) measurements were performed to assess endothermal transitions of collagen (DSC Q1000, TA Instruments, New Castle, DE, USA). Eight to thirteen mg of collagen dispersion was hermetically sealed in a T_{zero} pan (aluminum) and equilibrated at 1°C for 2 min. A temperature ramp was performed from 1 to 80°C, at 5°C/ min. An empty aluminum pan was used as the reference. Values determined included temperature at which helix to random coil transition of the collagen started (T_{onset}), temperature where 50% of the collagen has unfolded (T_{peak}), and the denaturation enthalpy (ΔH) using the DSC software (Universal Analysis 2000; Version 4.5A). Four replicates of each skin type were run in duplicate, whereby the average of the duplicates was used in the statistical analysis.

2.7 Rheology of collagen dispersions

The flow behavior of the different dispersions was investigated, as this could indicate the applicability of the dispersions for use in a co-extrusion system. The rheology measurements were performed based on Oechsle *et al.* (2016), with slight modifications. Measurements were conducted with an oscillatory rheometer (TA Instruments AR2000, New Castle, USA), which was equipped with a Peltier plate and a water bath. The collagen dispersions were analysed by oscillating measurements, using a 40 mm diameter plate-plate (hard-anodised aluminium) geometry. Stress sweeps were performed at 1 Hz applying an oscillatory stress from 0.01 to 1000 Pa at 5°C (linear viscoelastic range determination). Subsequently frequency sweeps were performed by applying 0.1% strain,

determined from the linear viscoelastic range, from 1 to 100 rad/s. Next, the complex viscosity η^* could be determined as a function of angular frequency ω by applying Eq. 1 (Macosko, 1994):

$$\left[\left(\frac{G''}{\omega}\right)^2 + \left(\frac{G'}{\omega}\right)^2\right]^{1/2} \quad \text{(Eq. 1)}$$

By plotting the complex viscosity η^* as a function of the angular frequency ω the dynamic consistency index k^* (Pa s^{n^*}) and the dynamic power law factor n^* (-) were calculated by applying Eq. 2 (Keogh and O'Kennedy, 1998).

$$\eta^* = k^* \omega^{n^* - 1} \quad \text{(Eq. 2)}$$

Temperature sweeps of the dispersion were performed in the linear viscoelastic range by applying 0.1% strain from 5 to 60°C at 2°C/ min at 1 Hz. The temperature at which G' (Pa) started to decrease (at least one decade) was determined as a measure of the helix to random coil transition. Four replicates of each skin type were run in duplicate, whereby the average of the duplicates was used in the statistical analysis.

2.8 Primary amine group content of collagen dispersions

The number of primary amine groups present in the lyophilized dispersions were determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS). The methodology originated from Buttafoco *et al.* (2006). Briefly, 1 mg lyophilized samples were incubated for 30 min in 1 ml aqueous solution of 4% w/v $NaHCO_3$. A solution of 0.5% w/v TNBS (1 ml in MilliQ water) was added to the mixture and incubated at 40°C for 2 h. After adding HCl (3 ml, 6 M), hydrolyzation started at 60°C for 90 min. A 96 well plate was used to dilute the mixture 1:1 with MilliQ water, followed by mixing and measuring the absorbance at 420 nm (Bio-Tek Spectrophotometer, Bad Friedrichshall, Germany). A glycine calibration curve was used to calculate the concentration of free amine groups. Four replicates of each skin type were run in triplicate, whereby the average value was used in the statistical analysis.

2.9 Film preparation from cattle skin collagen dispersions

Film forming ability, strength and Young's modulus of the films were evaluated by putting 5 g of each dispersion on a stainless-steel board, between two layers of plastic sheets, and flattening them with a stainless-steel roller (Fig. 3). To achieve uniform film thickness, a roller with a recess of 0.5 mm was used. Sodium chloride solution (24%) was added by a Pasteur pipette to the side of the collagen film, which was rolled flat between two plastic sheets. Then the collagen precipitated, by gently lifting the plastic sheet to the top of the collagen film. Ten films were made (dimension 150 mm x 40

mm) from each of the four replicates (per skin type) and evaluated for their mechanical properties.

Figure 3. Procedure for collagen film preparation. A) Collagen dispersion on a stainless-steel board between two layers of plastic sheets. B) Flattening of the collagen dispersion with a stainless-steel roller. C) Addition of saturated sodium chloride solution to the side of the collagen film, thereby gently lifting the plastic sheet. D) Collagen film precipitated with saturated sodium chloride solution.

2.10 Mechanical properties of collagen films

Films were determined by texture analyzer (TA.XT2, Stable Micro Systems, Godalming, UK) equipped with a 5 kg load cell to evaluate the Young's modulus and strength of the films after exposure to 24% NaCl (mimicking industrial settings). Therefore, the films were cut into 150 mm x 40 mm strips and precipitated in 24% NaCl solution. The tensile measurements were performed by fixing the film with tensile grippers, employing a crosshead speed of 40 mm/s, gripper initial distance was set at 20 mm, target distance

at 120 mm, and a break sensitivity at 1.0 N. The initial thickness (0.5 mm) and width (40 mm) of each film was used for the tensile stress calculations. Tensile strength (maximum stress the film can handle prior to breaking) and the Young's modulus (maximum elongation the film reached just prior to breaking) were determined from the created stress-strain curves. Ten films per batch were evaluated, and the average value was used in the statistical analysis.

2.11 Primary amine group content of crosslinked collagen films

Films were crosslinked overnight with 5% glutaraldehyde (Merck, Darmstadt, Germany), followed by lyophilization. The number of primary amine groups present in the lyophilized crosslinked collagen films was determined using TNBS as described. Four replicates of each skin type were measured in triplicate, whereby the average value was used in the statistical analysis.

2.12 Statistical analysis

In the experiment, different cattle types were used, each being slaughtered at a different age. This means that the cattle type was confounded with age. Therefore, only type was included as a factor in the statistical model.

Statistical analysis was performed (Minitab Version 19, Minitab Ltd., Coventry, UK). Data were checked for normality in both means and residuals. A general linear model was used for analysis of variance (ANOVA) for protein and soluble collagen content, thermal, rheological, and mechanical properties, and the primary amine group content. The model used was:

$$Y = \mu + Type + Run + e,$$

where Y = dependent variable; μ = overall mean; Type = American Calf (AC), Dutch Heavy Veal (DHV), Danish Ox/ Heifer (DOH), or Heavy German Cow (HGC); Run = 1, 2, 3 or 4; e = residual error. Data are presented as means ± SD. Means were compared after correction for multiple comparisons using the Bonferroni post-hoc test ($p \leq 0.05$).

Spearman correlation analysis was used to determine the relationship between soluble collagen content and viscoelastic and mechanical properties of both dispersions and films ($p \leq 0.05$).

3. Results and discussion

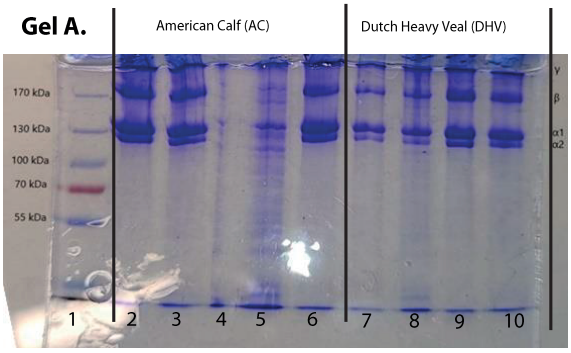
3.1. Co-extrusion technology: Zone 0

3.1.1 Collagen dispersion

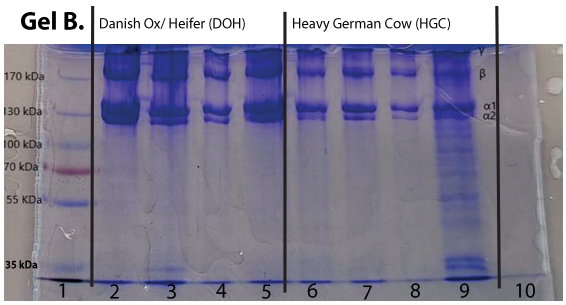
Zone 0 of the co-extrusion process relates to receiving the raw collagen dispersion and in our study to determine its composition and quality. Soluble collagen content is of importance for understanding the flow properties (i.e., providing the glue to hold the fibers and fibrils together). The amount of soluble collagen (tropocollagen) in the dispersion depends on the age of the animal and chemical processing. For example, collagen derived from young animal's skin has a high level of soluble collagen, and the liming process will also solubilize some of the collagen. The amount of soluble collagen in commercial dispersions is not always fixed and depends on processing conditions.

Soluble collagen extracted from the skins of HGC (1.6%) was lower ($P<0.05$) than for DOH (9.8%), AC (9.5%) and DHV (6.6%). It was expected that the soluble collagen content of HGC would be lower compared to AC and DHV since these animals are older. Miller *et al.* (1983) reported that with increasing the animal age, a lower soluble collagen content was found in corium samples. This is related to increased development of stable intramolecular and intermolecular crosslinks, which are more resistant to destruction by heat or acids (Miller *et al.*, 1983).

To produce collagen dispersions, type I collagen which is present in the bovine skin is used (Oechsle *et al.*, 2016). Type I collagen triple helix contains two α1(I) molecules and one α2(I) polypeptide chains (Chambers, 2015). The dispersions prepared from AC, DHV, DOH and HGC showed a double band at approximately 130 kDa (Fig. 4), probably indicating α1(I)- and α2(I)-chains of type I collagen (molecular mass of $\alpha_2 < \alpha_1$ with approximate mass ratio 1:2). In addition, a band above 170 kDa was present, which may indicate a β-chain. The dispersions prepared from DHV-R2 and DOH-R2 showed bands at 35 kDa, most likely pepsin used during preparation of the collagen dispersions. All other dispersions showed no low molecular weight bands, indicating no major presence of degraded collagen, except for samples AC_R3 and HGC_R4. Here bands were visible at lower molecular weight, indicating collagen breakdown products and/ or small protein contaminants. As these two isolations suffered from temperature built-up in the high shear mixer, it is most likely degraded collagen, resulting in heat denaturated collagen (gelatin). For this reason, it was decided to exclude dispersions AC_R3 and HGC_R4 from further analyses.



Lane	Sample and run (R) number
1	Prestained protein ladder
2	AC-R1
3	AC-R2
4	Empty
5	AC-R3
6	AC-R4
7	DHV-R1
8	DHV-R2
9	DHV-R3
10	DHV-R4



Lane	Sample and run (R) number
1	Prestained protein ladder
2	DOH-R1
3	DOH-R2
4	DOH-R3
5	DOH-R4
6	HGC-R1
7	HGC-R2
8	HGC-R3
9	HGC-R4
10	Empty

Figure 4. SDS-PAGE gel of the marker (A and B lane 1) and of the dispersions on gel A: American Calf (AC-R1 to AC-R4) (lanes 2 to 6); Dutch Heavy Veal (DHV-R1 to DHV-R4) (lanes 7 to 10). Gel B: Danish Ox/Heifer (DOH-R1 to DOH-R4) (lanes 2 to 5); Heavy German Cow (HGC-R1 to HGC-R4) (lanes 6 to 9), indicating the presence of β, α1 and α2 collagen bands, whereby AC-R3 and HGC-R4 showing bands at the lower molecular weight, most likely due to degraded collagen as during mixing a too high temperature was generated in the high shear mixer. DHV-R2 and DOH-R2 show bands at 35 kDa, indicating the presence of pepsin. The four lanes per animal type represent four replications.

Dry matter content was analyzed as it indicates about all the other ingredients present in the dispersion. In general, a commercial collagen dispersion consists of 4 to 10% collagen (Bueker *et al.*, 2016) and 0 to 10% of modifiers (Barbut *et al.*, 2020), resulting in dispersion with 3 to 25% dry matter (Kobussen *et al.*, 2000). Dispersions in the current study did not differ in dry matter content and averaged 8.2 ± 2.7, 6.7 ± 1.6, 6.6 ± 1.5 and 6.1 ± 1.4 g/100 g, for AC, DOH, HGC and DHV, respectively. The values of AC, DOH and HGC were somewhat higher than those of commercial bovine collagen dispersions reported by Barbut *et al.* (2020), where the dry matter ranged from 4.3 to 6.4%.

Protein content was analyzed since it provides information useful to control the texture characteristics of the sausage, e.g., first bite in sausage. In general, a higher protein content in the casing results in a stronger casing. Protein concentrations in the dispersions

did not show significant differences between cattle types (P=0.560) and averaged 24.2 ± 1.1, 23.4 ± 1.3; 23.0 ± 0.8 and 22.7 ± 3 g/ 100 g for HGC, DHV, DOH and AC, respectively. Expressed on a dry matter basis the protein concentrations averaged 1.9 ± 0.7, 1.5 ± 0.2, 1.6 ± 0.3 and 1.4 ± 0.3 g/ 100 g for AC, DOH, HGC and DHV respectively. They are different compared to reported commercial bovine dispersions ranging from 3.5 to 5.1% (Barbut *et al.*, 2020). This difference is probably due to the procedure to calculate the protein content. Barbut *et al.* (2020) used the Dumas procedure, whereas this study used a Lowry assay.

3.2 Co-extrusion technology: Zone 1

3.2.1 Counter rotating cone extrusion

Zone 1 of the co-extrusion process (Fig. 1) is dealing with shaping of the product, i.e., creating a casing as the sausage is being produced while a collagen dispersion is added to the sausage surface, i.e., to be later gelled in place (Suurs and Barbut, 2020). As some heat can also be generated within the nozzle head (due to friction of the counter rotating action of the nozzles), water-cooled nozzles are used. However, it is still important to know the transition temperature of the collagen dispersion from the helical to random coil form (i.e., transformation to gelatin due to breakage of hydrogen bonds between adjacent polypeptide chains of collagen molecules (Zhang *et al.*, 2006), since the mechanical strength of a film is critical for the process (i.e., expansion of the meat during heating) and the final product (sensory perception). Fig. 5 shows that in all the temperature profiles between 35°C and 40°C all dispersions started to display a rapid decrease in elasticity and the loss modulus, reflecting intact trimers (γ) of collagen turning into individual chains (α) or dimers (β) in the transition from helical to random coil (Zhang *et al.*, 2006). Barbut *et al.* (2020) reported a rapid decrease in elasticity between 30 and 45°C for commercial bovine collagen dispersions. Our findings confirm the range of onset temperatures also measured by DSC (Table 1).

Overall, DSC measurements allow to determine the helix-to-coil transition temperature of the dispersions (Horgan *et al.*, 1991). No significant differences in T_{onset} and T_{peak} were found between the different collagen dispersions, indicating that cattle type had no influence on the thermal properties of the dispersions. However, the current study indicates differences in the enthalpy values among the cattle types, with lower values for HGC than for AC, DHV and DOH (P=0.27). According to Schroepfer and Meyer (2017), the enthalpy corresponds to the number of hydrogen bonds in the triple helix that actually provides stability to the helix. Lower enthalpy values correspond to degraded collagen, while crosslinking increases the denaturation temperature and results in higher enthalpy values (Paul and Bailey, 2003). A higher enthalpy value for the DOH dispersion may be related to the animal's type/ purpose (e.g., meat/ milk production versus working animals), having more collagen development

due to excercise and the environment conditions where they were kept (e.g., outside). A low enthalpy value from HGC dispersions was not expected as German heavy cows were used for milk production and breeding purposes and the higher slaughter age and older animals have more intramolecular and intermolecular crosslinks (Miller *et al.*, 1983).

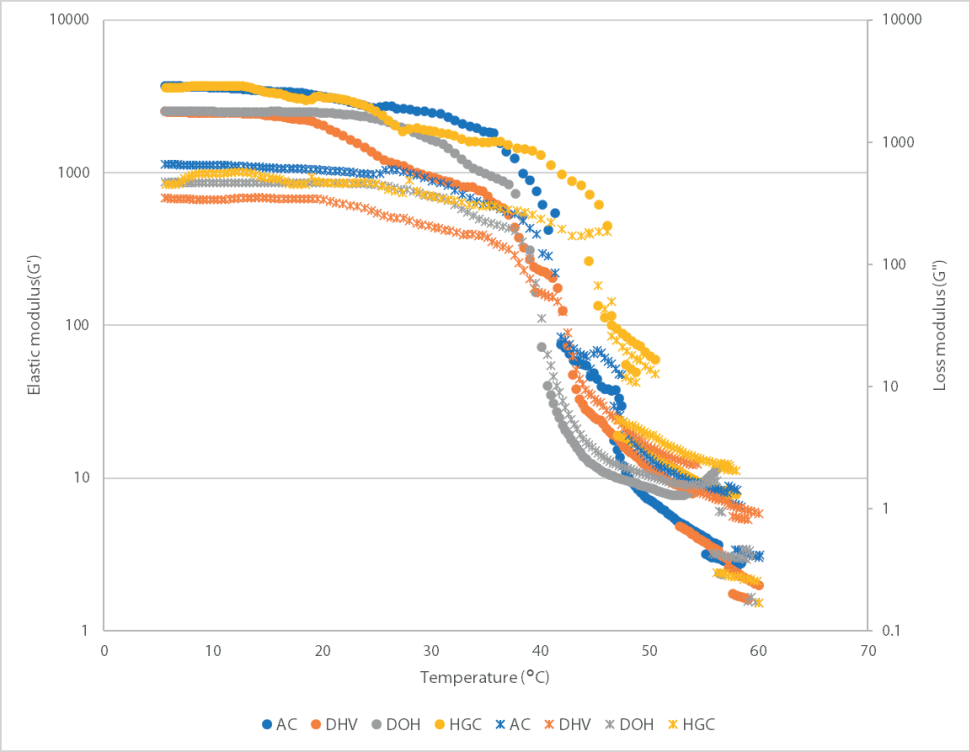


Figure 5. Representative curve of elastic (·) and loss modulus (x) (scans 5 to 60°C at 2°C/ min) of the cattle collagen dispersions: American Calf (AC); Dutch Heavy Veal (DHV); Danish Ox/ Heifer (DOH); and Heavy German Cow (HGC); showing for all dispersions a decrease in elasticity between 35 and 40°C (n=4 per treatment for DHV and DOH; n=3 per treatment for AC and HGC).

Table 1. Endothermic peaks analysis from the differential scanning calorimeter (DSC) thermograms cattle collagen dispersions made of American Calf, Dutch Heavy Veal, Danish Ox/ Heifer, and Heavy German Cow determined in duplicate as collagen dispersion (n=4 runs per treatment for DHV and DOH; n=3 runs per treatment for AC and HGC). Mean ± standard deviation.

Skin type	T_{onset} (°C)	T_{peak} (°C)	Enthalpy ΔH (J/g)
American calf	33.58 ± 0.31	36.34 ± 0.31	3.30 ± 1.10
Dutch heavy veal	33.38 ± 0.41	36.59 ± 0.79	2.82 ± 0.97
Danish ox or heifer	33.41 ± 0.45	36.21 ± 0.42	4.00 ± 1.27
Heavy German cow	33.52 ± 0.55	36.82 ± 0.66	2.32 ± 0.23
P-value_type	0.78	0.76	0.27
P-value_run	0.39	0.64	0.74

Based on the thermal stability in the current study, all collagen dispersions appear suitable to be used in a co-extrusion application, as this is in the temperature range reported for commercial bovine collagen dispersions.

3.2.2 Extrusion of the dispersion

The collagen dispersion should have the desired flow properties, i.e., it should be viscous enough that it flows out of the nozzle and forms a thin layer around the meat dough, and it must also be elastic enough so that the thin layer does not run off the sausages' surface. Determining the complex viscosity (η^*), dynamic consistency index (k^*), and dynamic power law factor (n^*) gives insight into these rheological properties of dispersions. All dispersions exhibited shear-thinning behavior, following a power-law model, as can be seen when the complex viscosity η^* is plotted as a function of the angular frequency (ω) (Fig. 6).

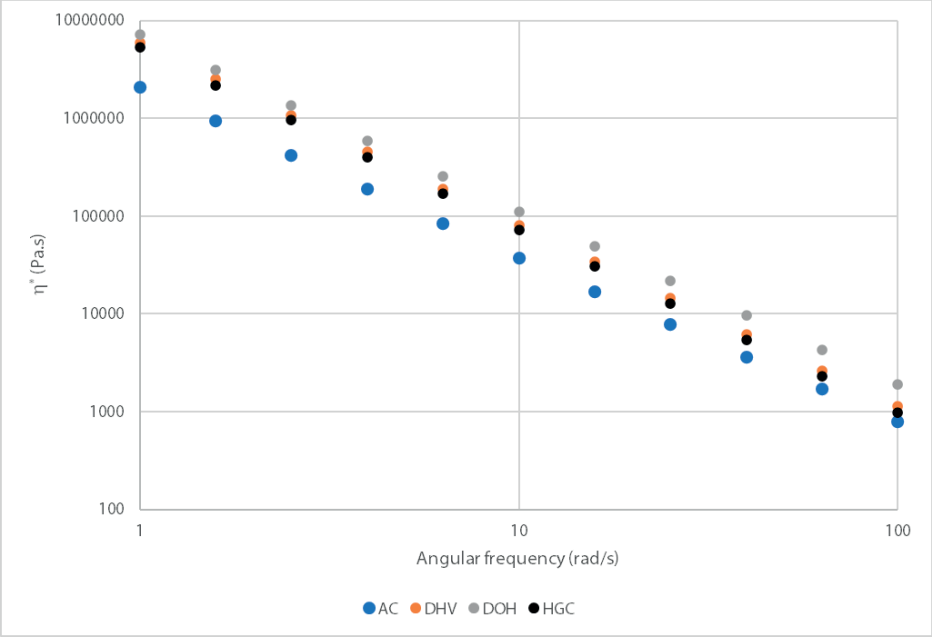


Figure 6. Complex viscosity η^* calculated from $\left[\left(\frac{G''}{\omega}\right)^2+\left(\frac{G'}{\omega}\right)^2\right]^{1/2}$ of cattle collagen dispersions made of American Calf (AC), Dutch Heavy Veal (DHV), Danish Ox/ Heifer (DOH), and Heavy German Cow (HGC) as a function of the angular frequency ω (n=4 runs per treatment for DHV and DOH; n=3 runs per treatment for AC and HGC). Overall, showing shear thinning behavior following a power-law model for all dispersions.

Overall, the complex viscosity of all dispersions was comparable. The k^* value showed no significant difference for cattle type (P> 0.05, Fig. 7A), but large variations in k^* were

found within the different cattle types, which may be due to variability of skin tissue within the cattle type used to make the dispersion. Skin parts located closer to the spine of the animal are tougher (stronger fiber bundles) than parts located closer to the abdomen (loose fiber bundles) (Covington, 2009). As tougher bundles will swell less compared to loose fiber bundles, this could result in differences in viscosities. The k^* values of HGC, DHV, AC, and DOH were 59, 68, 95 and 114 Pa s^{n^*}, respectively. In the production of commercial bovine collagen, large kneaders are used, including several homogenisation steps, which results in a smooth homogenous dispersion (Bueker *et al.*, 2016), unlike our protocol. Different acids and concentrations have an effect on the rheological behaviour of the dispersions. Hydrochloric acid for example is the preferred acid, used by the industry, as it yields a more viscous paste and improves the swelling rate of the collagen fibers (Ratanavaraporn *et al.*, 2008; Ioi, 2013; Sobanwa, 2021). Oechsle *et al.* (2014) reported that collagen entanglement and network formation depends strongly on the pH and acid type. They found enhanced collagen entanglement with increased ionic strength of the solvent (in case pH < pI). This can influence the production of the collagen dispersion, depending on what is needed for the co-extrusion process. According to Oechsle *et al.* (2014) it is likey that the highly entangled collagen matrices will probably result in co-extruded collagen casings with high elasticity and tensile strength.

The power law factor (n^*) (Fig. 7B) showed no significant difference for cattle type ($P > 0.05$). The n^* values for AC, DHV, DOH and HGC were comparable (0.02), which means they demonstrate an elastic behavior. Viscoelastic systems have a n^* value between C and 1, where 0 means completely elastic and 1 means Newtonian behaviour (Keogh and O'Kennedy, 1998). The values found in our study 0.02 ± 0.004, 0.02 ± 0.006, 0.02 ± 0.004 and 0.02 ± 0.007 (-) for AC, DHV, DOH and HGC, respectively are comparable to the commercial bovine collagen dispersions ($n^* = 0.01$).

Spearman correlation between soluble collagen content and viscoelastic properties of all dispersions showed a positive relationship ($p=0.009$) with the dynamic consistency index k^* ($r = 0.670$). Changes in soluble collagen content are associated with changes in the dynamic consistency index k^*. No relationship of soluble collagen content was found with the power law factor n^* ($r = 0.213$, $p = 0.464$).

Considering that not only the k^* value is responsible for flow properties of the dispersion, but the amount of soluble collagen as well (Barbut *et al.*, 2020), it appears that the HGC skin is less suitable as collagen source for co-extrusion casings. Moreover, it is expected that the flow properties of the dispersions prepared from AC, DHV and DOH skins are not optimal for co-extrusion, because of the high k^* values, and therefore require viscosity adjustment by dilution with aqueous acetic acid.

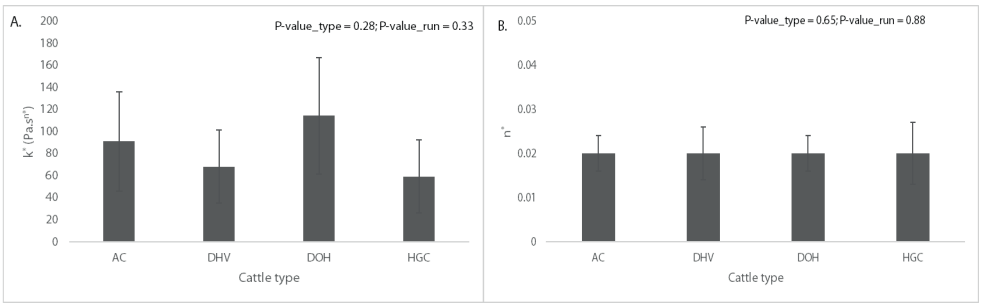


Figure 7. A) Dynamic consistency index (k^*) and B) the dynamic power law factor (n^*) of collagen dispersions made from AC (American Calf, DHV (DUTCH Heavy Veal, DOH (Danish Ox/Heifer) and HGC (Heavy German Cow). Bars represent mean ± standard deviation. A) Highest consistency value (k^*) was found for DOH, but no significant differences between the dispersions. B) all dispersions show almost elastic behavior. N=4 runs per treatment for DHV and DOH; n=3 runs per treatment for Ac and HGC.

3.2.3 Brining of the dispersion - creating mechanical strength

The next step in the co-extrusion process (Fig. 1) is creating mechanical strength by immersion of the sausage rope in a sodium chloride solution (e.g., 20 – 30% NaCl), which dehydrates the casing by water removal. This results in a denser collagen fiber network that provides the initial strength to the casing (Kobussen *et al.*, 2000; Visser, 2012). This is important as the sausage is later exposed to different stresses, as it undergoes subsequent treatments, like smoking, drying and cooking with additional moving and shaking on a conveyor, and pushing into baskets (Kobussen *et al.*, 2000; Morgan *et al.*, 1988). The sodium chloride solution doesn't affect the sensory properties of sausages produced under commercial conditions. As a matter of fact, the salt concentration at the sausage surface will be low as water is being pulled out of the product/ "expelled by the casing". Overall, in industrial co-extrusion applications salt must be added on a continuous basis to the circulation solution to keep it always saturated. The general observation was that films could easily be made from the AC, DHV and DOH dispersions, but films from HGC dispersions showed poor film forming ability, probably due to the fibrous structure of the dispersion resulting in films with small fractures. However, no significant differences (P>0.05) were found among the different skin types Ultimate Tensile Strength (UTS) values were 1.6 ± 0.4, 1.6 ± 0.9, 1.3 ± 0.5, and 1.2 ± 0.5 MPa, for AC, DOH, DHV and HGC, respectively; Fig. 8A. It was expected that the dispersion of HGC would yield weaker films (i.e., tensile strength), possibly due to the low soluble collagen content, which is necessary to hold the fibers and fibrils together. The amount of soluble collagen was low as the raw material originated from animals that have formed quite some crosslinks due to age (Miller *et al.*, 1983).

The Young's modulus of the films was calculated to gain insight into the behavior of the films when subjected to a force. A higher Young's modulus is associated with brittle films, whereas a low modulus reflects flexible films (Chakravartula, 2019). No significant differences (P>0.05) were found among the different skin types in Young's modulus and averaged 170 ± 92, 119 ± 30, 103 ± 51, and 83 ± 23 MPa, for DHV, HGC, DOH and AC, respectively; Fig. 8B. Large variations in strength and Young's modulus were found within the different cattle types, probably due to the large variation in parts of the skin being used to prepare the dispersions. Barbut *et al.* (2020) analyzed five commercial bovine collagen dispersions and reported tensile strength values that were approximately 6 times lower. This difference is most probably due to differences in exposure time in the NaCl. Barbut *et al.* (2020) used an exposure time of 5 min in 30% NaCl, whereas in this study maximal exposure time in 24% NaCl was applied (overnight storage of the films between two plastic sheets without rinsing, so high salt concentration remains on the surface) before measuring the mechanical properties.

Spearman correlation between the soluble collagen content and the texture properties of all the films prepared showed a negative relationship with the Young's modulus of the films (r = -0.512; p = 0.043). This means that a higher proportion of soluble collagen content results in a lower Young's modulus and thus a more flexible film. No relationship with soluble collagen content was found for the tensile strength values (r = 0.418, p = 0.107).

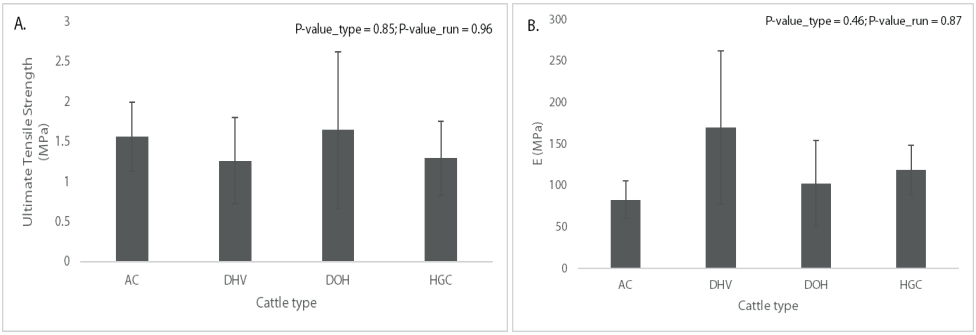


Figure 8. Mechanical properties of collagen films prepared from cattle dispersions produced by exposing to 24% NaCl; A) Ultimate tensile strength (UTS), and B) Young's modulus (E) for American Calf (AC), Dutch Heavy Veal (DHV), Danish Ox/ Heifer (DOH), and Heavy German Cow (HGC). Overall, showing no significant differences in UTS and E values for the films. n = 4 runs per treatment for DHV and DOH; n=3 runs per treatment for AC and HGC. Bars represent mean ± standard deviation.

Overall, from the film strength perspective it is preferred to use dispersions prepared from AC, DHV and DOH. Although the films from HGC dispersion could be evaluted for their mechanical properties, the film forming capacity of the HGC dispersion was not sufficient.

3.3 Co-extrusion technology: Zone 2

3.3.1 Crosslinking the films

For sausage manufacturers it is important to have information regarding the presence of crosslinkable groups in the dispersions and the final strength of the film as it is related to the sensory properties of the sausage. The TNBS assay was used to determine the free amine groups after crosslinking with glutaraldehyde. In this study, the choice was to use glutaraldehyde as crosslinker as its aldehyde content is known, so that it can serve as a reference for the efficiency of liquid smoke available on the market (Barbut and Ioi, 2019). Dispersions did not differ in the number of amine groups ($P>0.05$) and averaged 482 ± 32, 436 ± 53, 440 ± 34, and 439 ± 56 nmol/ mg, for HGC, AC, DOH and DHV, respectively (Table 2). Significant differences were expected between the younger AC and DHV versus older DOH and HGC (Miller *et al.*, 1983), since the AC and DHV skins were soaked in NaOH for 24 h, while for DOH and HGC skins this was 48 h. Longer time in high alkaline solution can result in higher reduction of NH_2 groups (Suurs and Barbut, 2020).

After crosslinking the films, with 5% glutaraldehyde, no statistical differences were observed between AC, DHV, DOH and HGC crosslinked films. AC films showed 324 ± 51 nmol/ mg free amine groups, whereas for DHV, DOH and HGC it was 355 ± 39, 324 ± 34, and 354 ± 40 nmol/ mg, respectively, corresponding to a reduction of 26, 19, 27 and 27% primary amine groups (Table 2). Considering the ability of the dispersions to create crosslinks with glutaraldehyde, it is obviously expected that liquid smoke will also be able to form crosslinks, and therefore all four dispersions are considered suitable to produce cooked smoked sausages by the co-extrusion technology.

Table 2. Number of NH_2 groups present in collagen dispersions and crosslinked films (5% glutaraldehyde) determined with TNBS assay. Dispersions and films were prepared from skins of American calf (AC), Dutch heavy veal (DHV), Danish ox/heifers (DOH), and heavy German cow (HGC). Overall, showing no significant differences in amine groups between the different dispersions and films. n=4 runs per treatment for DHV and DOH; n=3 runs per treatment for AC and HGC. Mean ± standard deviation.

Type	Amine groups in dispersion (nmol/ mg sample)	Amine groups in x-linked films (5% glutaraldehyde) (nmol/ mg sample)	Reduction of free amine groups
American calf	436 ± 53	324 ± 51	26%
Dutch heavy veal	439 ± 56	355 ± 39	19%
Danish ox/ heifer	440 ± 34	324 ± 34	27%
Heavy German cow	482 ± 32	354 ± 40	27%

4. Conclusions

The properties of the dispersions and films are summarized in Table 3. Skins of the AC, DHV and DOH were found to have the best potential to serve as a suitable collagen source for the co-extrusion process. HGC skin appears less suitable since its film forming ability was poor, which is an important property for co-extruded films. The study also showed that optimization of the preprocessing treatments appears to play an important role and that different skin sources may need to be treated differently to achieve the best suitability. In addition, soluble collagen in the dispersion is a factor to be considered as it influences the viscoelastic and mechanical properties of the dispersions and films.

Table 3. Properties of dispersions and films per skin type [American Calf (AC), Dutch Heavy Veal (DHV), Danish Ox/ Heifer (DOH) and Heavy German Cow (HGC)] and their performance with respect to co-extrusion process and/ or final product

Measurement	Properties in relation to co-extrusion process/ final product	American calf	Dutch heavy veal	Danish ox/ heifer	Heavy German cow
SDS-PAGE	Type I collagen	+	+	+	+
DSC	Helix-to-coil temperature	+	+	+	+
Rheology	Extrudability	±	±	±	-
Texture	Film forming capacity	+	+	+	-
Texture	Casing strength	+	+	+	-
Amine groups	Crosslinking potential	+	+	+	+

± = property of the dispersions/ films meets the criteria

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CHAPTER 5

5

Collagen dispersions prepared from porcine urinary bladder and intestine as raw material for natural co-extruded sausage casings

Short communication

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In preparation

Abstract

Natural casings, derived from sheep and hog intestines, are valued by consumers, because of their organoleptic properties, i.e., elasticity, tenderness and "snappy" texture properties when eating a sausage. A major disadvantage of these casings is their lack of uniformity in thickness, diameter, and length. Moreover, the sausage production process with natural casings is a batch process, which requires quite some manual labor. An alternative for these natural casings is co-extruded casings, which uses collagen dispersions derived from bovine hides. However, these casings cannot match the textural and cooking properties of natural casings. The current study investigated the application of porcine urinary bladder and intestines as an alternative collagen source for dispersions for co-extrusion with the aim to increase textural properties. Warner Bratzler shear force of the sausages produced with urinary bladder and combination of urinary bladder and intestine (ratio 90/ 10) ranged from 36 to 47 N. This is higher than the shear force values of sausages produced with commercial bovine hide collagen dispersion (28 – 35 N), but still lower than the values for the benchmark products produced with sheep casing (47 – 61 N). Moreover, the secondary cooking properties of sausages produced with casings from urinary bladder and intestine collagen appears to be better compared to the sausages produced with bovine hide collagen (less blistering). Considering the mechanical and cooking properties, collagen from porcine urinary bladder with and without porcine intestinal collagen have great potential for co-extruded sausage production.

1. Introduction

Natural casings represent a 50% share of the global casing market in 2021 and they are an essential part of many sausage products (Anonymous, 2021). The main source of edible natural casings are sheep and hog intestines of which the submucosa layer is used (Harper *et al.*, 2012; Rebecchi *et al.*, 2015; Gunn *et al.*, 2022). Consumers value natural casings, because of their unique organoleptic properties as they exhibit excellent elasticity and tenderness properties when consuming a sausage. During processing, they exhibit good permeability for smoke diffusion through the casing and into meat thereby enhancing the flavor. Natural casings also exhibit good thermal and mechanical stability (e.g., ability to shrink during cooking) and show good adhesion to the meat mass. All these properties result in the "snappy" texture perceived in cooked sausages such as frankfurters (Sobanwa, 2021; Gunn *et al.*, 2022). However, one of the disadvantages of natural casings is their lack of uniformity in thickness, diameter and in length (Sobanwa, 2021). Moreover, the sausage production is a batch type process, meaning that quite some manual labor is required during the production process. Therefore, new pre-manufactured casings have been developed from alternative sources, such as cellulose, regenerated collagen, and plastics to overcome some of these disadvantages. More recently, another technology for sausage casing application has been developed that can replace the traditional batch type processing for continuous manufacturing, namely co-extrusion technology. Hereby, the preparation and storing of pre-made casings is made obsolete by applying a collagen dispersion consisting of soluble and insoluble type I collagen fibers originating from the corium layer of bovine hides (Suurs & Barbut, 2020). This semi-liquid dispersion is introduced simultaneously with the meat batter, by a counter rotating cone system, and is gelled in place with the application of a saturated sodium chloride solution. However, the mechanical properties (e.g., tensile strength, stiffness) of casings formed with this collagen gel are less than those of pork/ beef/ sheep natural casings. Gunn *et al.* (2022) noted that the mechanical properties of casings made from natural pork/ beef/ sheep intestines depend on several collagen characteristics: content, fibril diameter, structural arrangement and the nature and extent of crosslinking. In particular, the "bite" characteristic (consumer impression) is lower compared to that of natural casings, probably due to the morphological differences between the natural and co-extruded collagen casing (Chen et al, 2019; Gunn *et al.* 2022). Chen *et al.* (2019) and Gunn *et al.* (2022) showed with SAXS that sheep casings consist of thick collagen fibers oriented at ± 45° from longitudinal direction with an interwoven network structure, whereas manufactured collagen casings show a fine distribution of fibrils with large gaps in between, leading to higher swelling ratio upon heating and therefore reduced mechanical properties. Chen *et al.* (2019) reported a significantly higher swelling ratio of the collagen film after heating compared to sheep casing. Co-extruded sausage casings may suffer from similar drawbacks as its manufac-

turing is comparable to the manufactured collagen casings. Researchers investigated methods to enhance the mechanical properties by exploring methods such as physical and chemical crosslinking and blending collagen with hydrocolloids, such as guar gums and derivatized celluloses (Chen *et al.*, 2019; Shi *et al.*, 2019 and Sobanwa, 2022).

In this preliminary study, we explored whether porcine raw materials, i.e., urinary bladder and intestines, enhance mechanical properties of co-extruded collagen casings. In addition, secondary cooking properties of these materials were examined, as the casing based on current commercial bovine collagen dispersion used for co-extruded sausages commonly shows poor secondary cooking characteristics (Sobanwa, 2022) (e.g., the casing peels off completely from the meat dough when reheating the sausage in a pan with hot oil).

The choice for utilizing bladder and intestines gives the opportunity to upgrade residual materials from animal processing operations. Consequently, utilized pieces of intestines that were removed from large segments of long intestines, because of small cavities and other short pieces were all used to make the collagen dispersion. Normally these pieces of intestine are "glued together" to form a stretch of long intestine, but this is a labor-intensive process, and the strength of these intestines portions is impaired. The urinary bladder is traditionally used in Southern-Europe as a casing for large diameter sausages or as an ingredient in traditional meals. By using the bladders to make a co-extrusion dispersion, the applicability of this material can be increased. The experiment compared the mechanical properties and secondary cooking characteristics of sausages produced with commercial bovine collagen dispersion and collagen dispersion prepared from the urinary bladder and intestines and combination of the latter two materials.

2. Materials and methods

The data described in this paper was collected over consecutive experiments over a 1-year time frame as each experiment was a developmental step (indicated by the date of the trial) in the process. This paper reflects the potential of the new raw materials as a source for co-extrusion sausage casings, based on mechanical and secondary cooking properties, but optimization of the dispersions and possible ratios between the different raw materials need further investigation.

2.1 Collagen dispersions

Different collagen dispersions were evaluated as raw material for co-extrusion casings, including experimental urinary bladder collagen (BC); combination of urinary bladder and intestine collagen (BIC), provided by AniMox (Berlin, Germany) and two commercial

bovine collagen dispersions (CC1 and CC2) manufactured by Devro B.V. (Gendt, The Netherlands). In addition, natural sheep casings (NSC) (Ø 24-26 mm) (van Hessen, Chicago, USA) were used as a benchmark.

The procedure for dispersion preparation was performed according to Noorzai *et al.* (2019) with modifications. Briefly, bladders were thawed, washed (1x), frozen followed by cut into pieces of < 2 cm with a knife. Bladder were incubated in 0.3 M NaOH (Sigma-Aldrich) at a sample:solution ratio of 1:3 (w/v – based on the wet weight f the original sample, 1 g of sample with 3 ml of solution). The mixtures were soaked for 40 h at room temperature. Thereafter, the bladders were washed 5 times with water, followed by mincing at 3 mm in a meat mincer (TPM 8, Cermenate, Italy). The minced bladder was put on a sieve to drain the water from the mass. Next step, the minced bladder were swollen by adding a mixture of 0.2 M lactic acid/0.2 M acetic acid/0.04 M hydrochloric acid (Sigma-Aldrich) at a sample:solution ratio of 1:1.5 (w/v) for 60 h. Finally, the swollen bladder dispersion was homogenized in an emulsifier (MC12, Stephan Food Processing Technology, Hameln, Germany), whereby the first milling was at 0.7 mm, followed by a run at 0.35 mm. The salted intestines were washed (3x), frozen followed by cut into pieces of < 2 cm with a knife. Intestines were incubated in 0.3 M NaOH at a sample:solution ratio of 1:3 (w/v – based on the wet weight of the original sample, 1 g of sample with 3 ml of solution). The mixtures were soaked for 40 h at room temperature. Thereafter, the intestines were washed 5 times with water, followed by mincing at 3 mm in a meat mincer (TPM8). The minced intestines were put on a sieve to drain the water from the mass. Next step, the minced intestines were swollen by adding a mixture of 0.2 M lactic acid/0.2 M acetic acid/0.04 M hydrochloric acid at a sample:solution ratio of 1:1.5 (w/v) for 62 h. Finally, the swollen intestine dispersion was homogenized in an emulsifier (MC12, Stephan Food Processing Technology, Hameln, Germany), whereby the first milling was at 0.7 mm, followed by a run at 0.35 mm.

2.2 Meat batter composition

Pork meat recipe was used to produce the sausages, consisting of 40% lean meat (90% meat/ 10% fat) and 44% fatty fraction (50% meat/ 50% fat), both ground at 4 mm (K+G Wetter, 130 meat grinder, Breidenstein, Germany). The lean fraction was mixed under vacuum (UM/VM44, Stephan Food Processing Technology, Hameln, Germany) for 3 min while adding water (14.5% w/v), sodium chloride (1.0% w/v), sodium triphosphate (0.3% w/v) and curing salt with nitrite (0.2% w/v). Next, the fat fraction was added followed by 2 min under vacuum. The meat batter was prepared two days in advance of sausage production.

2.3 Sausage preparation

A co-extrusion system equipped with a piston stuffer (Marel Townsend Further Processing, Boxmeer, The Netherlands) for the collagen dispersion, and a vacuum filler (Risco, RS110, Thiene, Italy) for the meat and extrusion head were used. Equipment was kept at 4°C at the test facility. The piston stuffer applied a constant pressure (5 bar) to the metering pump (56 Hz), which subsequently supplied the collagen to the extrusion head. The gel dispersion used mimicked the industry standards of 3 - 5%. The interchangeable extrusion head consisted of a 30 mm (inner Ø) extrusion head with independently counter rotating cones equipped with a mixing ring, whereby the outer cone speed was set at 391 rpm and the inner cone speed at 290 rpm. Collagen was pumped through the rotating extrusion head and in between the outer and inner cone, while being extruded through a 350 µm circular die. At the same time the meat dough was pumped through a pipe reaching the center of the extrusion head. Simultaneously as the collagen dispersion was leaving the circular die, the meat dough was extruded, so a thin layer of collagen dispersion was coated onto the meat dough. Immediately upon exiting the extrusion head, saturated sodium chloride (24% w/v) was applied onto the sausage rope to start hardening of the collagen and forcing water out of the coated gel. This was followed by a portioning step to divide the sausage rope into sausage links. Subsequently the sausage links were showered for 2 s with saturated sodium chloride (24% w/v) and submerged for 60 s in potassium lactate solution (58% w/v). Sausages were collected and dipped for 8 s in a 12.5% (w/v) liquid smoke solution (Red Arrow 24P, Smoky Light BV, Reeuwijk, The Netherlands). Sausages were then cooked in a hot air chamber (R&D FP, Marel Further Processing, Boxmeer, The Netherlands) by applying different heating steps and conditions. Step 1: Dry bulb temperature (T_{db}) = 74°C, absolute humidity (AH) = 5 g/ kg, and air velocity (V_{air}) = 3.5 m/s for 30 min. Step 2: T_{db} = 77°C, AH = 95 g/ kg, and V_{air} = 3.5 m/s for 15 min and step 3: T_{db} = 82°C, AH = 200 g/ kg, and V_{air} = 3.5 m/s for 15 min until core temperature (T_{core}) reached 72°C. Sausages were then cooled at 4°C, vacuum packed, and stored at 4°C for further analysis.

Sausages with different casing types were produced, whereby the bladder/ intestine collagen dispersion (BIC ratio 90/10) was tested in four different experiments (i.e., test dates), but the first three experiments (BIC_1 to BIC_3) used the same dispersion (A) and only in the last experiment (BIC_4) a new prepared dispersion (B) was used (Table 1). For the sausages produced with the bladder collagen (BC_1 and BC_2) two different dispersions (C and D) were used. Two commercial bovine collagen dispersions (CC1 and CC2) were used, whereby production with CC1 was repeated in two different experiments (E).

Table 1. Shows the different dispersions the dates of the sausage production and which dispersion was used (A to F). In case the same letter applies means that the sausages were prepared with the same dispersion

Dispersion	Test date	Dispersion
BIC_1 (ratio 90/10)	16-09-2021	A
BIC_2 (ratio 90/10)	27-09-2021	A
BIC_3 (ratio 90/10)	18-10-2021	A
BIC_4 (ratio 90/10)	23-05-2022	B
BC_1	18-10-2021	C
BC_2	24-05-2022	D
CC1_1	25-02-2021	E
CC1_2	18-10-2021	E
CC2	18-10-2021	F

2.4 Mechanical properties of the sausage

After 24 h at 4°C, sausage texture was evaluated by a texture analyzer (TA.XT2, Stable Micro Systems, Godalming, UK) equipped with a 30 kg load cell. Shear force of sausages was evaluated (at room temperature) using a Warner Bratzler shear cell with a V-cut blade. The shear measurements were performed by placing the sausages on a platform perpendicular to the slot length, where every sausage was sheared twice. The shear measurements were performed by employing a crosshead speed of 10 mm/s. The maximum force necessary to shear through the sausage was determined. Three sausages per casing type were measured three times and the average value of the three measurements was used in the statistical analysis.

2.5 Secondary cooking performance

After 24 h at 4°C, sausages were evaluated for their secondary cooking performance, simulating home preparation. Sausages were heated in a pan with 2 ml of hot oil (~180°C) and were turned every 1 min until internal cooking temperature (consumption temperature) was about 60 – 65°C (total cooking time 5 min). The degree of blistering of the casing was determined by visual grading as: minimal, medium, and high. Minimal is considered as less than 25% of the surface was blistered, medium is considered when 25 to 50% of the sausage surface was blistered and high is considered as more than 50% of the sausage surface was blistered.

2.6 Benchmarking – sheep casing

Benchmark products were made by stuffing the coarse meat dough (same recipe) in sheep casing diameter (Ø 24 – 26 mm) using a vacuum filler (Risco, RS110). Sausages were stuffed to their recommended stuffing diameter (Ø 26 mm), followed by cutting to

individual links, and drenching for 8 s in a 12.5% (w/v) liquid smoke solution (Red Arrow 24P, Kerry, Wisconsin, USA). Sausages were cooked in a hot air oven (Single Smoke Cart, Alkar oven, Lodi, USA) according to the basic program for sausages with collagen casing program for smokehouses (program supplied by the oven manufacturer). Sausages were cooked for 35 min at a T_{db} of 46°C with relative humidity of 72%, followed by cooking for 40 min at a T_{db} of 60°C and relative humidity of 40%. The final cooking step lasted 20 min with a T_{db} of 77°C and relative humidity of 100%, so that the internal temperature of the sausage reached 72°C. This was concluded with a cold shower (~15°C) for 15 min.

2.7 Statistical analysis

The statistical software Minitab Version 19 (Minitab Ltd., Coventry, UK) was used to perform a one-way analysis of variance (ANOVA; Tukey test; $p<0.05$) for the shear force. The model used for shear force was:

$$Y = \mu + \text{Gel dispersion} + e,$$

Where Y= dependent variable; μ = overall mean; Gel type = urinary bladder/ intestine (BIC) or urinary bladder (BC) or commercial bovine collagen (CC1 or CC2); e = residual error. Data are presented as means ± S.D. Three sausages per casing type were evaluated and the average of three measurements per sausage were used in the statistical analysis.

The secondary cooking characteristics of the sausages were not statistically assessed as this was a very subjective measurement and depends on the person rating the products.

3. Results and Discussion

The aim of the research was to investigate whether porcine under-utilized organs such as urinary bladder and smaller pieces of intestines (< 1 m) can be used as raw materials for co-extruded casing and enhance organoleptic properties ("snappy texture") to those of sausages stuffed in natural casings. Another research item was to assess whether these materials led to increased performance during secondary cooking (i.e., sausage heating/ frying at home).

The shear force value of the benchmark products produced with sheep casing was between 47 and 61 N (Fig. 1). This baseline was used to evaluate the sausages made with the new casing materials, i.e., porcine urinary bladder and intestines. Two commercial bovine collagen dispersions (CC1 and CC2), currently used by the industry (made from the corium layer of bovine hides) were included in the comparison. A significant difference

in shear force was found for the sausages produced with different dispersions (P<0.05) (Fig. 1). The sausages produced with dispersions prepared from BIC and BC exhibited the highest shear force with 45 ± 5 and 47 ± 3 N, respectively. The sausages produced with commercial bovine collagen dispersion (CC1) exhibited the lowest shear force of 28 ± 2 N. The shear force values of the commercial bovine collagen dispersion ranged from 28 to 35 N, lower than the range of the benchmark products. Sausages produced with dispersions prepared from urinary bladder (BC) and a combination of urinary bladder and intestines (BIC) (ratio 90/ 10) resulted in higher shear force values ranging from 36 to 47 N, closer to the range of the benchmark products. The shear force data showed quite some variation within a dispersion type over different production dates. This variation can be related to fluctuations in the meat mass, differences in meat mass preparation (i.e., standing time of the meat mass), protein content of the casing (variation due to new batch of collagen gel or the amount of gel dispersion applied to the meat batter), or fat exudation from the meat mass during cooking, resulting in fat pockets on the sausage surface. The fat pockets on the sausage surface influence the texture measurements negatively, as the casing is pulled from the meat batter by the fat.

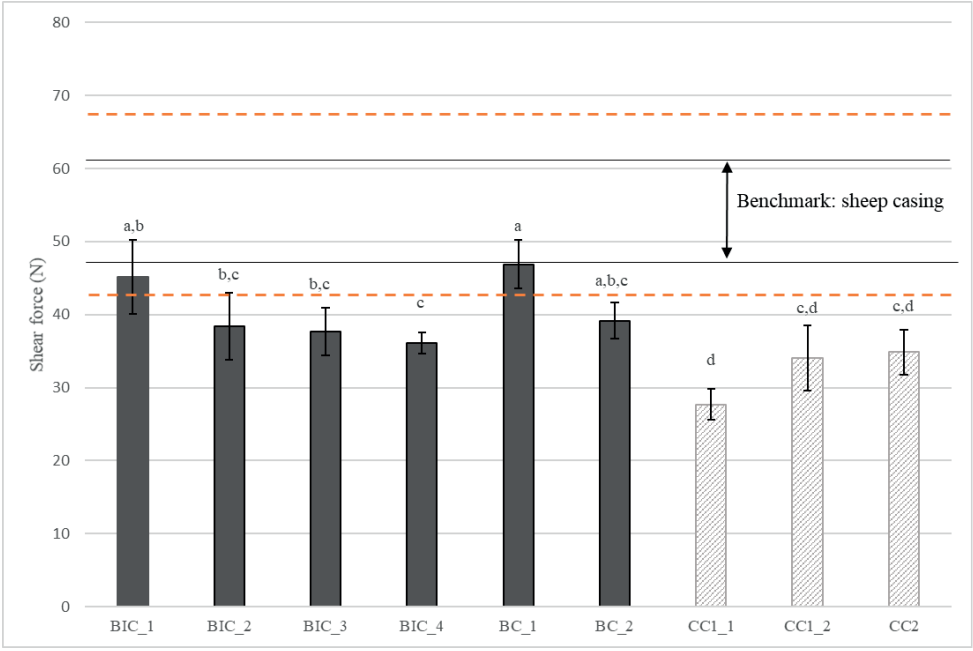

Figure 1. Shear force values of sausages prepared from, bladder/ intestine collagen (BIC_1 to 4: ratio 90/10), bladder collagen dispersion (BC_1 and BC_2), commercial bovine collagen dispersion (CC1 and CC2. Overall, showing that BIC_1 and BC_1 reach the benchmark value of natural casing sausages (orange dotted line represents standard deviation of sheep casing). Bars represent mean ± standard deviation. P-value_dispersion type = 0.000.
a,b,c,d - bars with the different letters means significant difference

Attempts were made to prepare sausages from a 100% intestine dispersion, but this was not possible as the dispersion was too fibrous for extrusion. Savic and Savic (2016) mentioned that the collagen fibers in intestinal tissue are strong, insoluble, and bound in a complex network. Furthermore, collagen fibers may be crosslinked to a larger extent both inter- and intramolecularly as the animal ages. Intestines also contain a high proportion of elastic fibers (Savic and Savic, 2016), which makes it more difficult to handle as a raw material for dispersions. The elastin content of hog casing is 5.5% (Bakker *et al.*, 1999). Alternatively, the bladder seems to be a suitable collagen source as the fibrils are found in all layers of the bladder's wall (mucosal and serosal layer), and therefore appear to be easier to process into a flowable dispersion, which may also be due to the lower elastic fibers content of the bladder (1.2% for human urinary bladder) (Murakumo *et al.*, 1995; Cortivo *et al.*, 1981). In addition, the bladder appears to be a good collagen source for making a dispersion for co-extrusion as the wall of the bladder is comprised of smooth muscle fibers consisting of actine and myosin known as the detrusor muscle (Tasian *et al.*, 2010) that might serve in the dispersion as the continuous phase for embedding collagen fibrils.

The secondary cooking properties of a sausage are an important aspect of the development as it determines how consumers will appreciate the new product and whether they will repeat their purchase. It is a common knowledge in the industry that the secondary cooking characteristics of sausages prepared with commercial bovine collagen dispersion are not as good as sheep casings (Sobanwa, 2021). The problem is that the casing can be peeled off upon reheating of the sausages. Once collagen is extracted from its natural material, it always shows inferior properties such as mechanical strength and lower thermal stability, due to unavoidable damage of the hierarchical structure, according to Xiao *et al.* (2020). We observed that sausages prepared with commercial bovine collagen scored high regarding blistering (Fig 2.). Our study showed that the mechanical and thermal properties can be improved to some extent by using a casing made from a dispersion of porcine bladder collagen (Fig. 2). The adhesion of the casing to the meat batter during cooking was much better than for commercial collagen gels and the thermal stability seemed improved as the overall degree of blistering was minimal, comparable to natural casing sausages (Fig. 2).

Figure 2. Representative picture of sausage produced with natural sheep casing, commercial bovine collagen casing, bladder/ intestine casing (ratio 90/10), and bladder casing after secondary cooking, showing minimal blistering (indicated by arrows) for sausages produced with bladder/ intestine casing and bladder casing, and high blistering (indicated by arrows) for sausages produced with commercial bovine collagen.

4. Conclusions

Based on the results of this preliminary study, it can be concluded that by using porcine's urinary bladder, whether or not in combination with its intestines, product characteristics comparable to sheep casing can be generated and thereby surpass the current performance of the currently used beef hide source. Optimization of the gel dispersion, ratios between bladder and intestines are necessary to match the shear force value of the benchmark product.

Acknowledgements

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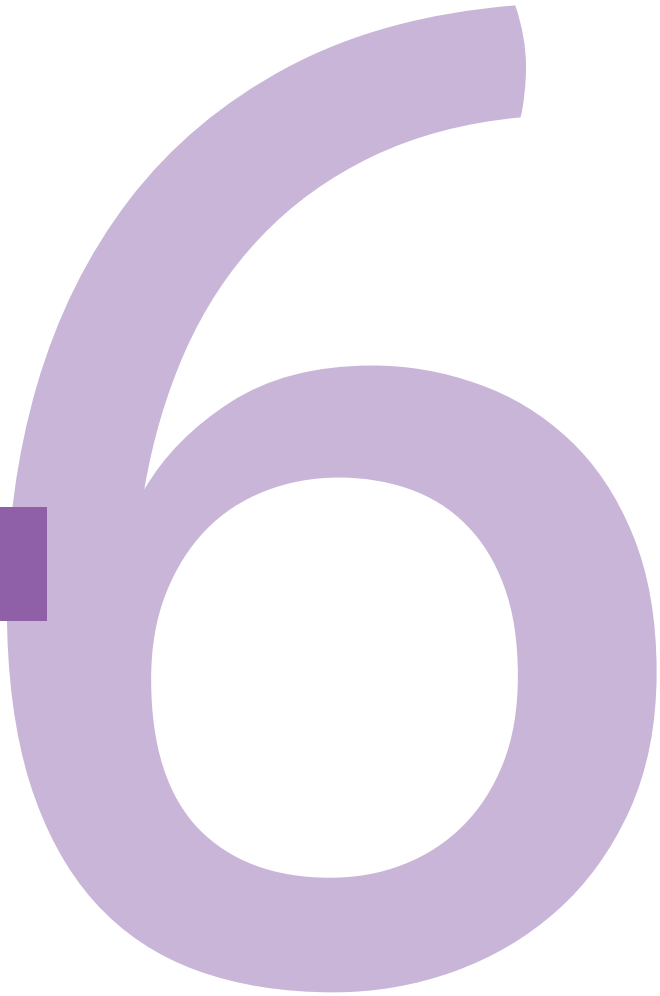
CHAPTER 6



General discussion

1. Introduction

Traditionally, sausages have an important place in our diet. This is due to their nutritional density, shelf life, unique texture, and flavor (Savic and Savic, 2016). The traditional process for sausage preparation is stuffing meat into sheep, pig and cattle intestines, known as natural casings (Wijnker, 2009; Adzaly *et al.*, 2015; Savic and Savic, 2016). The function of a casing is to accommodate sausage manufacturer's quality and processing needs. The ideal sausage casing should provide sufficient strength to undergo processing, while remaining tender enough during consumption (Miller *et al.*, 1983). Diseases, such as bovine spongiform encephalopathy, transmissible spongiform encephalopathy and foot and mouth disease, have caused the food industry to look for alternatives for natural casings. Besides, over the past two decades, a shortage and price rise of natural casings has put pressure on collagen casings and consequently sausage and casing manufacturers have searched for alternatives for sausage casing production. Moreover, the increased demand for kosher/halal products has only stimulated the search for alternatives (Barbut, 2010; Oechsle, 2016). Alternatives for natural casings are premanufactured casings, made from e.g., cellulose and collagen. Just like natural casings, these casings are stuffed with meat batter. Another alternative casing material is a collagen dispersion, that can be applied directly on a stream of meat batter using co-extrusion technology, whereby the casing is being formed at the moment the sausage is being produced. The big advantage of co-extrusion technology is the hygienic, and high volume sausage production, which results in consistent quality characteristics of sausages. Currently, bovine collagen from steer skins aged 18-36 months is the primary source for co-extruded casings. However, other raw materials from animal processing operations are available, such as intestines, bladder, and other parts of skins, which are now underutilized as they are considered as waste or are being processed in the food industry for other purposes. When using these alternative raw materials, collagen producers need data regarding the role of the biological factors, such as species, age, and breed on the collagen quality considered for casing production.

The aim of this thesis was to provide fundamental knowledge to understand effects of biological factors on collagen quality of livestock animals for the production of co-extruded casings. Three aspects were considered:

A. animal species: poultry – (Chapters 2 and 3), cattle – (Chapter 4) and pig – (Chapter 5);
B. collagen origin: skin – (Chapters 2, 3 and 4), bladder and intestine – (Chapter 5);
C. breed/strain: poultry – (Chapters 2 and 3), cattle – (Chapter 4).

To investigate these aspects, biochemical and mechanical characteristics of collagen dispersions and/or films related to the co-extrusion process were measured, including

extrudability, quality of the dispersion, temperature stability, film strength, and crosslinking potential.

The results of the four experimental chapters in this thesis are summarized in Tables 1, 2 and 3 showing the investigated biological factor, the properties of the dispersions and/or films per resource type and their potential performance with respect to the co-extrusion process and/or final product. Tables 1 and 2 show for Chapters 2, 3 and 4 whether the dispersions and associated films exhibit the properties (rated as plus [+] or minus [-] or plus/minus [±]) suitable for co-extrusion application. In the evaluation of the dispersions and films, the emphasis was on performance, assessed by rheology, texture measurements and temperature stability. The dispersions need to have a good extrudability, i.e., they need to be viscous enough to flow out of the nozzle head, but have a certain elasticity, meaning that the dispersion does not run off the sausage surface. Reference for this were the dynamic consistency index (k^*) and the dynamic power law factor (n^*) values of commercial bovine collagen, whereby the notation [+] means that the dispersion meets the indicated reference value of commercial bovine collagen. The notation [±] for extrudability means that the dispersion's value meets either the k^* or n^* value of the reference. The notation [-] means that the dispersion does not meet the indicated reference value of commercial bovine collagen. Next to extrudability, film strength is also of importance, as the casing dictates the initial sensory perception when eating/ biting into the sausage. In this case a notation [+] means that the film can be handled when clamping it within the grips of the texture analyser and thus can be measured. Finally, temperature stability of the dispersions needs to be at least comparable to commercial bovine collagens (T_{onset} ~ 34.5°C). In this case notation [+] means that the dispersion meets at least the indicated value of commercial bovine collagen. For the crosslinking potential, the notation [+] means that the dispersion meets the indicated reference value of commercial bovine collagen. As the lower limit for crosslinking is unknown, values lower than the indicated reference receive the notation [±].

Table 1. Effects of the biological factor strain/age of chickens and cattle, investigated in Chapters 2 and 4, on the properties of the dispersions and films (per skin type), and their potential performance with respect to the co-extrusion process and/or the final product.

	Factor: strain/age		Age	Dispersion composition (Presence of type I collagen)	Temperature stability (T_{onset} > 34.5°C)	Extrudability (k^*-value: 54 Pa s^{n^*} and n^*-value: 0.01)	Film forming capacity (Visual absence of holes/ fractures)	Film strength (Ability to clamp in grippers)	Crosslinking potential (> 284 nmol NH_2/mg sample)
Chapter 2	Chicken skin	Fast growing broiler	42 days	+	+	±	+	+	±
		Slow growing broiler	56 days	+	+	±	+	+	±
		Broiler Breeder	365 days	+	+	±	-	-	±
		Laying hen	728 days	+	+	±	-	-	±
Chapter 4	Cattle skin	American Calf	6 – 8 months	+	+	±	+	+	+
		Dutch heavy veal	6 – 8 months	+	+	±	+	+	+
		Danish ox/ heifer	max. 30 months	+	+	±	+	+	+
		Heavy German cow	30 – 60 months	+	+	-	-	-	+

[+] = property of the dispersions/films meets the criteria
[±] = property of the dispersions/ films meets some of the criteria
[-] = property of the dispersions/ films does not meet the criteria
Dispersion composition: presence of type I collagen
Temperature stability: T_{onset} > 34.5°C (compared to commercial bovine skin collagen)
Extrudability: k^*-value: about 54 Pa s^{n^*} and n^*-value: about 0.01
Film forming capacity: the dispersion must be able to roll out well into a thin film, without the presence of small holes and/or fractures
Film strength: Minimum requirement is that the the film can be handled when clamping within the texture analyser's grips
Crosslinking potential: commercial bovine collagen > 284 nmol NH_2/mg sample, but as the lower limit for crosslinking is unknown, values lower than the commercial bovine collagen are indicated with ±

Table 2. Effects of the biological factors strain and weight of broiler chickens, investigated in Chapter 3, on the properties of the dispersions and films (per skin type), and their performance with respect to the co-extrusion process and/or the final product.

	Factor: strain/ weight		Weight	Dispersion composition (Presence of type I collagen)	Tempe-rature stability (T_{onset} > 34.5°C)	Extrudability (k^*-value: 54 Pa s^{n^*} and n^*-value: 0.01)	Film forming capacity (Visual absence of holes/ fractures)	Film strength (Ability to clamp in grippers)	Crosslinking potential (> 284 nmol NH_2/mg sample)
Chapter 3	Chicken skin	Fast growing broiler	1.6 kg	+	+	±	+	+	±
		Slow growing broiler	1.6 kg	+	+	±	+	+	±
		Fast growing broiler	2.2 kg	+	+	±	+	+	±
		Slow growing broiler	2.2 kg	+	+	±	+	+	±

[+] = property of the dispersions/films meets the criteria
[±] = property of the dispersions/ films meets some of the criteria
[-] = property of the dispersions/ films does not meet the criteria
Dispersion composition: Presence of type I collagen
Temperature stability: T_{onset} > 34.5°C (compared to commercial bovine skin collagen)
Extrudability: k^*-value: about 54 Pa s^{n^*} and n^*-value: about 0.01
Film forming capacity: The dispersion must be able to be rolled out well into a thin film, without the presence of small holes and/or fractures
Film strength: Minimum requirement is that the the film can be handled when clamping within the texture analyser's grips
Crosslinking potential: Commercial bovine collagen > 284 nmol NH_2/mg sample, but as the lower limit for crosslinking is unknown, values lower than the commercial bovine collagen are indicated with ±

Table 3 summarizes for Chapters 2, 3, 4 and 5 the potential applicability of raw materials as a source for preparing collagen dispersion for co-extruded sausage casings (rated as plus [+] or minus [-] or plus/minus [±]) suitable for co-extrusion application, based on the information provided in Tables 1 and 2. The notation [+] means that the collagen source can be used as a raw material to prepare collagen dispersions. The notation [±] means that the source can be used, but the quality of the dispersion depends on the factors age, breed/strain and extraction process. The notation [-] means that the source is not suitable as a raw material.

Table 3. Overall suitability of different collagen sources, investigated in this thesis, as raw materials for dispersion preparation.

	Factor: collagen source	**Suitability as raw material for preparing collagen dispersion for co-extruded sausage casings**
Chapters 2 + 3	Chicken skin	**±**
Chapter 4	Cattle skin	±
Chapter 5	Pork bladder	+
Chapter 5	Pork intestine	-
Chapter 5	Pork bladder and intestine	+

± = property of the collagen source meets or don't meet the criteria: Suitability of the collagen source is based on the summary of the data presented in Tables 1 and 2 appearing in Chapters 2, 3 and 4, whereby the emphasis was on the performance; assessed by the rheology, texture measurements and temperature stability. The suitability of the raw materials for Chapter 5 are determined based on their performance in the co-extrusion process (the ability to form a fully cooked sausage).
[+] means that the collagen source can be used as a raw material to prepare good (or acceptable) collagen dispersions for co-extruded sausage casings.
[±] means that the source can be used, but the quality of the dispersion depends on the factors age, breed/strain and pretreatment process
[-] means that the collagen source is not suitable as a raw material to prepare collagen dispersions for co-extruded sausage casings.

Overall, it can be concluded that porcine bladder and intestine are a good collagen source for the co-extrusion application, however the latter is only suitable in combination with the bladder. In addition, the poultry and cattle skin can be used as a source, but the dispersion quality depends on the age and breed/strain of the animal, the extraction process, and equipment used to prepare the dispersion.

In the following section, the collagen dispersion and film properties required for the co-extrusion process will be discussed as well as how they are influenced by various factors.

First the influence of "age in relation to animal breed" on collagen quality for co-extruded sausage casing will be discussed. This discussion will be based on the soluble collagen content, extrudability, film strength, thermal stability, and crosslinking potential of the collagen dispersions/films.

Second, the influence of "animal species" on collagen quality for co-extruded sausage casing is discussed based on SDS-PAGE, film strength, and thermal stability.

Thirdly, the influence of "origin of the collagen" on the collagen quality for co-extruded sausage casing is discussed based on extrudability, film strength and temperature stability and crosslinking potential.

In each of these sections, a conclusion on the influence of the involved factor on collagen quality will be drawn. Finally, the effects of pre-treatment processes on the different variables will be discussed.

After discussing the influence of the indicated factors based on the different variables, a section will follow in which the relationship among different variables is examined, followed by a section on the availability of alternative collagen sources. Finally, the conclusion will follow and opportunities for further research will be discussed.

2. Age in relation to animal breed

In the experiments described in Chapters 2 and 4, different poultry and cattle breeds were used and each slaughtered at a different age. This means that in both chapters breed was confounded with age. Therefore, the age and breed of the animals will be discussed together in this section.

2.1 Dispersion composition

The "animal age" showed that the presence of crosslinks (i.e., increases with age of the animal) (Miller *et al.*, 1983), influenced predominantly the variables extrudability, and texture of the films (incl. film forming capacity). The more the native collagen is crosslinked, the lower the amount of soluble collagen in the collagen dispersion (Pines *et al.*, 1996; Yamauchi *et al.*, 1988). Soluble collagen is necessary for good extrudability and texture of the films, based on the data of Chapter 3 and 4. The correlation analysis in Chapter 4 showed a positive relation between the soluble collagen content and k^* value and a negative relation between the soluble collagen content and Young's modulus. A low amount of soluble collagen content was observed in the lower intensity of soluble collagen for dispersions prepared of older animals on SDS-PAGE in comparison with the

ones prepared from younger animals, which may indicate more crosslinked collagen in skins of older animals (Miller *et al.,* 1983; Pines *et al.*, 1996; Yamauchi *et al.*, 1988). For collagen dispersions from cattle skins of different ages, this difference was not seen on the SDS-PAGE (Chapter 4). When looking at collagen dispersions from broiler skins only, no differences in soluble collagen bands were observed on SDS-PAGE either (Chapter 3), which may be due to the small age difference in broiler chickens. Overall, collagen gel for co-extruded casing consists of fibrous and soluble collagenous material and this is also what we were investigating here (Barbut *et al*, 2020).

2.2 Extrudability

As we currently know, it seems that a certain amount of soluble collagen is needed for good extrudability of the dispersion. In the case of commercial dispersions, it is known that the amount of soluble collagen is not always fixed and also depends on processing conditions. However, the range is not a public knowledge. The dispersion prepared from porcine intestines (Chapter 5) showed poor film-forming properties that could be caused by the low content of soluble collagen, resulting in improper extrudability. The amount of soluble collagen was not determined in that case and therefore is now recommended to be measured in the future (Chapter 8). The data of the soluble collagen content in the other samples (measured by precipitation of the filtrate from the swollen skin material in a final NaCl concentration of 2.6 M, followed by dialysis) discussed in Chapters 3 and 4 revealed that in both poultry and cattle the amount of soluble collagen (in the skin) decreased as the animal ages. This means that the collagen dispersions from heavy German cows, broiler breeder and laying hens were less suitable for the co-extrusion process as the extrudability would not be ideal. This is expressed in the k^* value, which was lower for the dispersions prepared from older animals compared to the younger ones. The collagen swelling was less in skin dispersions from older animals (broiler breeder and laying hen – Chapter 2; heavy German cow – Chapter 4), probably because of more crosslinking, resulting in a lower k^* value.

2.3 Film strength

The soluble collagen content also seemed to be a critical parameter for the film forming capacity and film strength. The film forming capacity is an important aspect for the texture measurements, since the films were produced on lab scale (with a stainless-steel roller) before texture measurements were possible. Both poultry (Chapters 2 and 3) and cattle skins (Chapter 4) dispersions of older animals (laying hen and heavy cows) showed reduced film forming capacity compared to dispersions prepared from collagen obtained from young animals, resulting in small fractures in the film. The dispersion probably had a more fibrous structure, which may be a consequence of the more crosslinked collagen. In addition, the animal's purpose (e.g., meat/ milk production versus working animals) having more collagen development due to exercise and hous-

ing conditions (e.g., inside or outside) could played a role as well. The soluble collagen content was only measured for cattle skin dispersions, but the texture results showed a negative relationship between soluble collagen content and the Young's modulus. It can be speculated that this relationship also applies to poultry and porcine dispersions, meaning that for all species it was likely that as the animals ages, the proportion of soluble collagen decreases and Young's modulus increased to form more brittle films. In the case of the cattle dispersion prepared from the heavy German cows, film forming properties were poor and texture measurements could not be performed.

Next to soluble collagen content, collagen fibril diameter can also play a role in the film strength. The differences in film strength between poultry and cattle films can be related to the difference in collagen fibril diameter, as it is known that fibril diameter increases with age of the animal (Miller *et al.*, 1983; Wells *et al.*, 2013). Wells *et al.* (2013) reported that fibril diameter varies with strength, with several studies finding larger diameter collagen fibrils present in stronger tissue. Moreover, the fibril diameter may also be dependent on glycosaminoglycan content (Parry *et al.*, 1982). Genetic and environmental factors, such as breed of the animal, amount of fat, feed type and exercise level may also contribute to fibril diameter (Wells *et al.*, 2013). The diameter of collagen fibrils varies from 10 – 500 nm, depending on the locations of the tissues as well as the age and species of animal (Miller *et al.*, 1983; Parry and Craig, 1984; Wells *et al.*, 2013). Collagen fibril diameter for cow skin was found to be 67 nm, but also values of 142 – 163 nm in diameter are found. Whereas fibril diameter of 21-days old chicken was found to be 49 – 54 nm (Fleischmajer *et al.*, 1983). The diameters determined by TEM can greatly vary depending on sample preparation procedures and fixation methods. Cattle skin dispersions prepared from skins of younger animals produced films that were about 25 times stronger than the ones produced from the poultry skin dispersions. This large difference in film strength is probably not due to differences in fibril diameter as these values can differ as mentioned by Wells *et al.* (2013). Probably the protein content of the dispersion played a role, which was for the poultry dispersions ranging from 6.6 to 10% (Chapters 2 and 3) versus protein content of cattle dispersions ranging from 22.7 to 23.4% (Chapter 4).

2.4 Thermal stability

Miller *et al.* (1983) reported that crosslinking of the skin increases as animal ages. This should result in increased denaturation temperature and higher enthalpy values in our samples as more energy is necessary to break hydrogen bonds (Paul and Bailey, 2003; Schroepfer and Meyer, 2017). However, the dispersions from poultry (Chapter 3) and cattle skin (Chapter 4) showed that the different ages of the animals had limited or no significant influence on the denaturation temperature and enthalpy values. A trend in the enthalpy value was observed between dispersions prepared from veal calves, heif-

ers/oxen, and the heavy cows (Chapter 4), showing the highest enthalpy value for heifers/oxen, followed by veal calves and heavy cows. These differences were probably related to the animal's type/purpose (e.g., meat/milk production versus working animals), having more collagen development due to exercise, age and housing conditions (e.g., inside vs. outside). These differences resulting from factors such as excersise, were less explicitly present in the chickens, probably because excersise and housing did not differ that much between the different types used in the experiment. Differences in poultry might have been found if collagen was extracted from chickens that, for example, were grown in enriched environments with sand bathing and elevated feeding troughs.

The denaturation temperature of the collagen films was measured for the poultry dispersions (Chapters 2 and 3) and not for the cattle dispersions. The thermal stability of the films prepared from the younger poultry skin dispersions was not significantly different from older poultry skin dispersions, except for the ones prepared from laying hen skins. The films prepared from the laying hen skins showed the highest thermal stability. The films produced from older animals (broiler breeder and laying hen; Chapter 2) showed two helix-to-coil transformations (i.e., first transition at 57.9°C and second at 69.7°C for broiler breeder, and 61.8°C and 70.1°C for laying hen), which may be the lysine-aldehyde derived crosslinks that are replaced by mature crosslinks in the collagen as the animal ages (Miles *et al.*, 2005). Considering that an age effect existed for the films prepared from the poultry skin dispersions and that it is likely to be comparable to the films prepared from the cattle skin dispersions, it can be said that the quality of the film was probably influenced by the age of the animal, the older the animal, the higher the thermal stability.

2.5 Crosslinking potential

Information on the number of free amine groups gives meat processors the possibility to adjust texture properties of the casing. In the co-extrusion process, casings are crosslinked by applying liquid smoke, containing aldehydes that react with the collagen free amine groups. In general, the more free amine groups, the stronger casing will be. With respect to the age of the animal (within a species), no significant differences were found in the number of free amine groups. However, the expectation was to find differences, but in Chapter 2 no differences were found in free amine groups between the young broiler chickens and the older chickens (broiler breeder and laying hen) and this also held for the cattle skin dispersions (Chapter 4). No scientific data is available on the minimum amount of free amine groups necessary to obtain good/ acceptable crosslinked films suitable for co-extruded casings. This value cannot be deduced from the data of the heavy German cows, given that the dispersions from this source produced poor films. According to TNBS assay, sufficient free amine groups were available to crosslink (482 nmol NH_2/ mg sample versus 439 nmol NH_2/ mg sample for Dutch

heavy veal). Moreover, by adding glutaraldehyde, a decrease in free amine groups (27%) was found comparable to the films prepared from the dispersion of Danish ox/heifers skin. The consequence is that meat processors do not know what film strength to expect and therefore have no tools to control the texture of the casing/sausage.

2.6 Conclusion concerning the factor "age" in relation to breed

The factor "age of the animal" was found to be important in the extrudability of the dispersion, film forming capacity and the mechanical strength of the film.

3. Species

Species used in this thesis affected collagen type obtained, temperature stability, and film strength of the dispersions. Regarding the film strength, only the results of Chapters 3 and 4 will be discussed, considering that the production process and evaluation of the films described in Chapter 2 differed from those described in Chapters 3 and 4. After making the films described in Chapter 2, it was decided to optimize the dispersion production, since it appeared to be not fully homogeneous, and therefore made it more difficult to prepare the films.

3.1 Dispersion composition

The poultry (Chapters 2 and 3) and cattle skin (Chapter 4) collagen dispersions showed a banding pattern on the SDS-PAGE that were typical for type I collagen, suggesting that the major component of the collagen dispersions was made up of type I collagen. A Western blot could provide more insight in identifying collagen types by immunostaining the specific collagen types. It was expected that type III collagen is also present in the animal skin (Abedin and Riemschneider, 1984). Looking at the ultrastructure of the bladder wall (Chapter 5), it begins with urothelium, which is positioned upon the *lamina propria*: an extracellular matrix which serves of nutritive and informative support for cells of the *lamina propria*, composed mostly of type I and III collagen. Another type of extracellular matrix is found in the muscular layer of the bladder, where an elastic fiber network can be found (Bouhout *et al.,* 2013). In the interstitial matrix of intestines, collagen types I and III are the most representative subtypes both responsible for providing tensile strength to the organ tissues (Pompili *et al.*, 2021). These references indicate that the porcine dispersion thus also contained different fibrous collagen types. Additionally, in the experiment described in Chapter 5, it was possible to form a strong casing with the bladder/intestine material. For a good quality collagen dispersion, a fibrous collagen, preferably type I collagen, should be present; i.e., comparable to commercial bovine collagen dispersions. For industrial extruding of the collagen dispersion a counter rotating cone is required to orient the fibrils/fibers in a specified direction for

providing better mechanical strength of the casing (Hoogenkamp *et al.,* 2015). Thus, for good quality collagen dispersions, poultry and cattle skins from different breeds, and porcine bladder and intestines could be used as raw material. The quality of the dispersion, in respect to the materials used in this thesis, was not affected by animal species, but depended on the collagen source used for dispersion production.

3.2 Film strength

Differences in film strength were observed between films prepared from poultry (Chapter 3) and cattle skin (Chapter 4) dispersions. The collagen films prepared from cattle skin dispersions (except heavy German cow) were approximately 25 times stronger than those prepared from the poultry skin dispersions. As mentioned, this may be related to the difference in fibril diameter between poultry and cattle collagen. Miller *et al.*, 1983 and Parry and Craig, 1984 reported that the diameter of collagen fibrils varies from 10 – 500 nm, depending on the locations of the tissues as well as the age and specie of the animal. With respect to Young's modulus, the films prepared from poultry skin dispersions gave 24 times higher values than those from the cattle skin dispersions (except heavy German cow). This means that the films prepared from the poultry skin dispersions could yield more brittle films compared to the cattle skin dispersions, because a higher Young's modulus is associated with brittle films (Chakravartula, 2019), whereas a low modulus reflects more flexible films. In sausage production, this could lead, together with the lower film strength, into interruptions in production as the films could break more easily (in the industry known as "sausage rope breakage").

Although differences in strength and Young's modulus were large between the two species, the results of texture measurements (Chapters 3 and 4) showed that skins from both species were suitable as raw material, except the dispersions prepared from skins of older animals (cattle skin). The threshold of this value was set at the extent to which it was possible to manually handle the films and clamp them within the texture analyzer's grippers. Commercial bovine collagen was not taken as a reference, as a weaker film could also be suitable for sausage production, depending on the type of product (e.g., sausage with low initial "bite") and the meat processor preference.

Since poultry skins were found to be a suitable source, it must be taken into account that these films were weaker compared to cattle skin collagen. This may be reflected during the production of sausages, e.g., more breakage of the casing if the drying and cooking settings are too harsh, and the final product characteristics, i.e., by initial "bite" of the sausage. The "bite" perception will be less pronounced for sausages encased in chicken skin collagen compared to sausages produced with commercial bovine skin collagen. In any case, it depends on the meat processor preference and the market for which the products are prepared.

The collagen dispersions prepared from porcine materials (Chapter 5) resulted in strong films. However, whether the film strength of this dispersion was comparable or stronger than that of the poultry (Chapters 2 and 3) and/or cattle skin dispersions (Chapter 4) could not be judged since the texture of the porcine films was measured in whole products together with the meat batter; (as a sausage product) and not like the lab scale procedure performed for the poultry and cattle films. Considering the texture values obtained by the Warner Bratzler shear method, the strength of the porcine film dispersions (urinary bladder and/or intestines) were good, and almost comparable to natural sausage casings.

3.3 Thermal stability

The difference in temperature stability could originate from the differences in amino acid composition that varies per each specie, fibril thickness, crosslinking density, etc. (Hall and Reed, 1957; Parry and Craig, 1984). In Chapters 2, 3, and 4, results showed differences in transition temperature between poultry and cattle skin dispersions. Both the temperature sweeps measured (with the rheometer) and the T_{onset} (temperature in which protein denaturation starts) showed a higher denaturation temperature in case of the poultry skin dispersions (Chapters 2 and 3) compared to cattle skin (Chapter 4). This can probably be attributed to the two times higher lysine content (Gojkovic *et al.*, 2014), and the higher proportion of hydroxyproline in poultry skin compared to cattle skin (Paul and Bailey, 2003; Covington, 2011; Nik *et al.*, 2014). Lysine provides thermal stability to the collagen structure and is also involved in side-chain interactions. Denaturation temperature of the collagen triple helix depends on the number of hydroxyproline residues localized in the third position of the collagen helix (Burjanadze, 1982).

Differences were also observed in enthalpy values between poultry (Chapters 2 and 3) and cattle skin dispersions (Chapter 4). Cattle skin dispersions required more energy to break the hydrogen bonds versus the ones in the poultry skin dispersions. This may be attributed to the differences in fibril thickness and/or crosslinking density of the fibers in the skins or other factors related to the age differences between the two species. The thermal transition temperature was not measured for the porcine dispersions (Chapter 5), but the dispersions and corresponding films showed good thermal stability in the co-extrusion process. This was reflected in the film that withstood the forces of the expanding meat batter, during the heating process. In addition, the collagen film, on the final product, showed no signs of gelatinization as would be indicated by a sticky surface. The collagen dispersions and films should preferably have a comparable denaturation temperature to that of commercial bovine collagen dispersions, which Barbut *et al.* (2020) reported to be between 30 and 45°C.

3.4 Crosslinking potential

Differences in the number of free amine groups were observed between the dispersions prepared from poultry and cattle skin. Poultry skin dispersions (Chapters 2 and 3) showed lower number of free amine groups compared to cattle skin dispersions (Chapter 4). Although according to Gojkovic *et al.* (2014), the number of lysine residues in poultry collagen is to be two times higher than in cattle skin and can therefore in theory crosslink more. The protein content of the poultry skin dispersions (Chapters 2 and 3) was lower compared to cattle skin dispersion (Chapter 4). Protein content of poultry skin dispersions investigated in Chapter 2 ranged from 7.4 to 15.8%, in Chapter 3 a range of 5.2 to 7.4% was reported, and for the cattle skin dispersions a range of 22.7 to 24.2% was found, meaning lower protein content could have resulted in lower free amine content.

3.5 Conclusion concerning the factor "animal species"

The factor "animal species" was found to be important in the thermal stability (enthalpy) of the dispersion and mechanical strength of films.

4. Collagen origin

The origin of the collagen showed an influence on the extrudability, film strength and temperature stability of the collagen dispersions. Origin of the collagen means from which tissue/organ and from which location the collagen is isolated.

4.1 Extrudability

This part involves the skin's location on the animal that was used for collagen production. The influence of this on the extrudability was visible when comparing the viscosity data of the cattle skin dispersions (Chapter 4) versus the poultry skin dispersions (Chapter 2). A broad range of viscosities (59 to 114 Pa s^{n*}) was measured for the cattle skin dispersions, whereas the range for poultry skin dispersions was much smaller (35 to 41 Pa s^{n*}). It can be speculated that the broader range of viscosities was probably caused by the fact that the experiments were done with skin trimmings obtained from the leather factory, and it was unclear from which part the cattle skin were used. The production of commercial bovine collagen employs the parts that have the stronger fiber bundles, obtained from ox/steer skin of 18 – 36 months of age. Markiewicz *et al.* (2007) demonstrated that castration resulted in decreased skin thickness and collagen content in mice skin. It could be possible that this also applies to ox/steer skin and therefore the skins might be easier to process by collagen manufacturers. It is known that cattle can be raised inside or outside and this influences the collagen structure of the skin (Savic and Savic, 2016). For poultry (Chapter 2) the origin of the skin tissue was of less

importance, i.e., whether the breast or the back skin of the chicken is used, as these animals are generally kept indoors. Differences in thickness of the fiber bundles, and the location of the skin tissue probably influenced the degree of swelling of the cattle skin collagen dispersions (Chapter 4). The thicker bundles would swell less compared to smaller fiber bundles. In the current study, no viscosity measurement was determined for porcine urinary bladder/intestine dispersion (Chapter 5), and consequently, it is difficult to discuss whether or not the porcine collagen source influenced the viscosity of the dispersions.

4.2 Film strength

The results of the sausage texture measurements showed that a good firm film could be formed with the porcine urinary bladder/intestine dispersions. The elastin content of poultry skin is 4% (Kamaruzaman *et al.*, 2022), whereas for hog casings and the human pediatric urinary bladder, the content is 5.5% and 1.2%, respectively (Cortivo *et al.*, 1981; Bakker *et al.*, 1999). We anticipate that porcine urinary bladder will have a similar 1.2% elastin content. Overall, the addition of elastin probably yielded a stronger sausage texture compared to sausages produced with skin dispersions (Chapter 5). Bakker *et al.* (1999) mentioned that it is likely that the elastic tissue (5.5%) and the blood vessels network in the intestine contributes to its strength. Consequently, there is a chance that the raw material source played a role in obtaining good quality dispersions.

4.3 Thermal stability

Several studies mention that the denaturation temperature of the collagen triple helix depends on the number of hydroxyproline residues localized in the third position of the collagen helix (Burjanadze, 1982; Paul and Bailey, 2003; Covington, 2011). Hall and Reed (1957) reported that other factors, such as age may also be important in the thermal stability of collagen. The higher the proportion hydroxyproline, the higher the denaturation temperature of collagen. Given that the hydroxyproline content of the different dispersions was not measured and that denaturation temperature of pig dispersions were not measured, it is an opportunity for further research to investigate this relationship. The hydroxyproline content probably differs between locations of tissues, species and with the animals' age (Burjanadze, 1982).

4.4 Crosslinking potential

The major difference between the sources (i.e., skin, urinary bladder and intestines) was the presence of elastin, which may vary among tissue origin (Cortivo *et al.*, 1981; Bakker *et al.*, 1999; Kamaruzaman *et al.*, 2022). Elastin, present in the skin, bladder and intestines, also contains lysine residues and therefore the possibility exists that the porcine dispersion has more potential groups available for crosslinking as Bakker *et al.* (1999)

found that the elastin content of intestine is 5.5% versus 2 – 4% is skin. However, the values for elastin can vary as it is difficult to determine due to its high insolubility.

4.5 Conclusion concerning the factor "collagen origin"

It appears that the collagen origin has an influence on the collagen quality of the dispersion with respect to extrudability, film strength and crosslinkability and is therefore a factor to be considered. As different collagen sources could yield different film textures (depending on the wishes of the sausage producer) in terms of texture properties of the sausage, different raw materials can be used as a collagen source, e.g., such as skin, bladder, and intestines.

5. Pre-treatment process

The k^* value reflects the extrudability of the poultry and cattle dispersions and showed that this value was higher for the cattle skin collagen dispersion (Chapter 4) compared to the poultry skin collagen dispersions (Chapters 2 and 3). Even within the species, differences in k^* values were observed (Chapters 2 and 3). Although the collagen extraction procedure was comparable for poultry and cattle, many parameters differed between the species such as cleaning, cutting, liming, defatting, acid swelling and homogenization. For poultry collagen, the modified procedures of Munasinghe *et al.* (2014), described in Chapter 3, and Munasinghe *et al.* (2015) described in Chapter 2 as well as for cattle collagen (Chapter 4) the modified procedure of Noorzai *et al.* (2019) were used. The modified procedures of Munasinghe *et al.* (2014, 2015) differ from Noorzai *et al.* (2019) in dwell times in NaOH solution and acetic acid concentration. The adjustment, both for the method (extraction; chemical/dwell time/concentrations) and the equipment for homogenization (e.g., multiple homogenization steps, static and mechanical operations) can improve the extrudability (k^* value comparable to commercial bovine collagen) and film forming capacity of the skin dispersions. For the factor "age" was found that probably a reduction of the number of crosslinks in the skin could improve the dispersion quality with respect to extrudability and film forming capacity. Especially for the of older animals for both poultry and cattle (broiler breeder and laying hen – Chapter 2; Danish ox/heifer and heavy German cow – Chapter 4). By using a longer liming process for the skins of older animals, and partial hydrolysis of collagen, the ability of the fibers to swell in acetic acid solution will increase (Hood, 1987; Miller *et al.*, 1983). This may bring the k^* value of the dispersions in the range of bovine collagen dispersion and improve the film forming capacity and probably film strength. Furthermore, longer time in the lime solution could yield higher soluble collagen content and could therefore improve the extrudability. It can be speculated that if pretreatment procedures would be adapted to the maturity of the collagen and/or the animal's breed

(meat/milk production versus working animal) then improved values could be obtained from these animal resources as well.

The liming time could also influence the number of free amine groups, as a lower free amine group was found in poultry skins that were soaked for longer time in NaOH (Chapter 2). On the one hand, this probably resulted in a higher reduction of free amine groups in the dispersion (Hood, 1987), leading to lower values of free amine groups and probably crosslinking potential. On the other hand, due to hydrolysis more NH_2 groups could be formed if an amide bond interacted with water molecules to give a carboxylic acid (-COOH) and an amine group (-NH_2). Considering that no scientific data is available on collagen dispersions and the number of free amine group in these dispersions, it is difficult to estimate the lower limit of free amine groups that should be present to obtain an acceptable crosslinked film. This is an opportunity for further research to investigate the effect of liming time on the crosslinkability of the dispersions.

Extension of the lime process might be a better option than increasing the lime concentration as this could result in a less controllable process (faster chemical reactions, chance that too much collagen gets hydrolyzed or big differences within a batch, and functionality of the fibers is lost), longer time in a lower lime concentration would be a better controllable process step. However, an optimum in the liming process versus the desired k^* value must be found, especially if skins from older animals will be used, regardless of the animal species. This also accounts for the origin of the collagen. Extracting collagen from thicker fiber bundles of cattle skin probably requires longer liming times. The optimal time should be investigated in view of the effect of the lime solution on the cleavage of NH_2 groups that are necessary for crosslinking (Hood, 1987).

In addition, different homogenization step is a variable to consider as this differs from commercial steer skin collagen preparations. The type of homogenization (i.e., equipment) could influence the extrudability of the dispersions. The *k** value was found to be higher for the cattle skin collagen dispersion (Chapter 4) compared to the poultry skin collagen dispersions (Chapters 2 and 3). Even within the species, differences in *k** values were observed (Chapters 2 and 3). Moreover, the *k** value for the cattle skin dispersion was higher compared to the two commercial bovine collagen dispersions. The poultry dispersion in Chapter 2 was prepared with a food processor (Stephan, UMC5), whereas in Chapter 3 a blender (JAP blender) and in Chapter 4 a high shear mixer (Marel) was used to prepare the dispersions. Moreover, the porcine dispersion was produced with a colloid mill (Stephan, MC12). This could have caused the differences in *k** value between and within the different species, as different forces were exerted on the dispersion (more homogeneity with colloid milling) and resulting in different fiber lengths (Bueker *et al.*, 2016).

Acid swelling is also a factor to consider in the pre-treatment process. The cattle skins were swollen in 0.7 M acetic acid versus 0.5 M acetic acid for poultry skins. Oechsle *et al.* (2014) reported that collagen entanglement and network formation depends strongly on the pH and acid type. They found enhanced collagen entanglement with increased ionic strength of the solvent (in case pH < pI). This can influence the production of the collagen dispersion.

5.1 Conclusion concerning pre-treatment process

The pre-treatment process should be considered as an important tool to control the influence of the "age of the animal", "species" and "collagen origin" of the animal factor on dispersion/film quality.

6. Relationships among studied variables

Table 4 shows the variables measured to study the influence of the biological factors on quality of the collagen dispersions. Spearman correlations ($p \leq 0.05$) were made between the investigated variables to find possible relationships. Correlations are indicated if they yielded significance for both the individual dispersion (e.g., poultry – Chapter 3 or cattle – Chapter 4) and the pooled data. Table 3 shows that a negative relationship exists between the ultimate tensile strength and Young's modulus ($r= -0.959$; $p \leq 0.001$). This means that a high ultimate tensile strength results in a low Young's modulus; e.g., a strong, but flexible film is obtained. Furthermore, a positive relationship was found between the ultimate tensile strength and k^*-value ($r = 0.773$; $p = 0.000$), meaning that changes in the k^*-value are positively associated with changes in the ultimate tensile

Table 4. Relationship among the studied variables of collagen dispersions prepared from poultry and cattle skins.

	Protein content	**Ultimate tensile strength**	**Youngs modulus**	**Free amine groups**	***k**** **value**
Protein content		-	-	-	-
Ultimate tensile strength	-		r = -0.959 p= 0.000	-	r = 0.773 p= 0.000
Youngs modulus	-	r = -0.959 p= 0.000		-	-
Free amine groups	-	-	-		-
k* **value**	-	r = 0.773 p= 0.000	-	-	

- means no correlation found

strength. With the two significant relations, it is possible to choose which measurement method to use. Particularly for the positive relationship between k^*-value and ultimate tensile strength a choice can be made for one of the two methods. Looking at the industrial applications, it is more likely that they have the availability of a texture analyser over a rheometer. In this case, when measuring the ultimate tensile strength of the films, the k^*-value can be calculated.

7. Availability of alternative collagen sources

7.1 Bladder dispersion

Considering the results of this thesis, the question arises whether or not the availability of the certain by-products (skins, porcine urinary bladder and intestines) are available in sufficient quantities for collagen producers. In 2021, The Netherlands processed 1.4 million broilers, 47,000 pigs, 5,800 cattle and 4,200 calves per day (CBS, 2021). If viewed from a European perspective, the total number of pigs slaughtered is many folds higher. An average sausage producer is processing about 2,500 kg sausage per hour x 16 production hour, resulting in 40,000 kg sausage per day. For this production, 3 – 5% collagen dispersion is needed, requiring 1,200 – 1,500 kg dispersion per day.

From a dispersion production point of view (Chapter 5 of the thesis), it is known that about 60 kg of bladder (raw material) results in 90 kg collagen dispersion (factor 1.5; i.e., due to the addition of diluted NaOH the 60 kg raw material will swell, and the total weight will be about 90 kg). For producing 1,200 – 1,500 kg dispersion, 800 to 1,000 kg bladders will be needed per day (1,200/1.5= 800). If each bladder weighs 50 g (Anonymous, 2023) then 20 bladders are required to get 1 kg. If 1,200 kg of dispersion is needed per manufacturer, 16,000 to 20,000 bladders are required per day (to supply one sausage producer with the collagen dispersion). Based on the number of slaughtered pigs from the CBS (2021), and the estimation for the European volume, there seem to be enough pig supply to fulfil the bladder demand for dispersion production.

7.2 Intestine trimmings

From a dispersion production point of view (Chapter 5), it is known that about 40 kg of raw desalted intestines results in 80 kg collagen dispersion (factor of 2). For 1,200 – 1,500 kg dispersion, 600 to 750 kg intestines/day are needed (1,200/2=600). In 2012, one Chinese factory processed annually about 1.5 billion meters of natural intestines (Van der Plas, 2012). Assuming that 1% are trimmings (pieces of intestines < 1 m) this equals 15 million meters of natural casing trimmings, that are currently not utilized, and therefore available for collagen dispersion production. The weight of these trimmings

(< 1 m) equals to 15 g (Anonymous, 2023). Therefore, 15 million m x 15 g = 225,000,000 g equals 225,000 kg/ year of casing trimmings. To produce 1 kg intestine 66.67 x 15 g are required. If 600 kg per day are required, about 40,0 kg/ day of intestines are required to fulfil the demand for dispersion production. There seem to be enough intestine trimmings available to fulfil the demand for dispersion production.

7.3 Skin trimmings

A prominent environmental biologist dr. Senior (University Kent, Canterbury, United Kingdom) recently mentioned in an interview (Vandoorne, 2023) that on a yearly basis the leather industry deals with > 8-million-tons of animal skins. Assuming 1% are skin trimmings that end up as waste, this means that 80,000 tons of skins are available for sausage casings dispersion production. Assuming that the conversion factor from skin to dispersion is also 1.5 (i.e., that 1 kg skin results in 1.5 kg of usable dispersion), these trimmings will result in 120,000 ton of collagen dispersion. Thus, with an average utilization of 1,200 to 1,500 kg of dispersion per day per manufacturer, this should be more than enough for supplying the collagen producers.

8. Conclusions

Table 5 summarizes how collagen quality for the co-extrusion application is influenced by the biological factors investigated in this thesis. Table 5 shows that all investigated factors influence collagen quality. Particularly age in relation to breed as well as the collagen source are important. However, collagen extraction process appears to be the key factor in obtaining a good quality collagen dispersion and later the film. According to the results of the thesis, different skin sources (influenced by collagen origin and animal breed) need to be treated differently to achieve the best suitability.

Table 5. Overview of how collagen quality, for co-extrusion purposes, is influenced by biological factors and the extraction process investigated in this thesis.

	Species	Age related to breed		Collagen origin	Pre-treatment process
Extrudability	-	+		+	+
Dispersion composition	-	+		±	+
Temperature stability	+	± (dispersion)	± (films)	+	+
Film strength	+	+		+	+
Crosslinking potential	-	-		±	+

[+] means that the biological factor/extraction process influences the variable that contributes to the quality of the dispersion
[±] means that the biological factors/extraction process influences partially the collagen quality
[-] means that the biological factor/extraction process does not influence the variable that contributes to the dispersion quality

Based on the information presented in Table 5 the main question stated in the introduction of the thesis can be answered:

How do biological factors of livestock production influence the quality of the collagen matrix?

All investigated species (poultry, cattle, pig) and their different collagen sources (besides cattle skin) appear to be suitable to produce dispersions suitable for co-extrusion application. The older the animal, the less suitable the sources are to produce dispersions for the co-extruded sausage casings.

9. Future research opportunities

This thesis provides fundamental knowledge for understanding the effects of biological factors, related to livestock animals, on collagen quality for the production of co-extruded casings. However, not all questions have been answered. Thus, the following research opportunities are still open:

- Investigate the collagen extraction procedure. Determine the optimal preparation procedures for different collagen sources. Examining factors influencing the extraction procedure, such as dwell times in NaOH solution, NaOH concentration, pH, acid type, etc.
- Investigate the minimum amount of soluble collagen and primary amine groups required for crosslinking collagen dispersions/films to obtain a good quality collagen dispersion. Also investigate the relations of raw material's age and the associated collagen extraction procedures (may differ with collagen source).
- Perform biochemical and microscopical analyses of the urinary bladder/intestine dispersions to provide better understanding of the texture properties as compared to currently used bovine skin collagen.
- Upscaling of porcine urinary bladder/intestine dispersion to optimize pre-treatment process so that the dispersion can be industrially produced
- Study how amino acid composition (including hydroxyproline), of the dispersions relates to species differences and its influence on the thermal stability and crosslinking potential of collagen dispersions.

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CHAPTER S

Summary

Summary

Sausage casings are an essential component in the transformation of comminuted meat into a finished product. The strength of the casing, together with the texture of the meat, determines the sensory perception "bite" when presented to the consumer. Traditionally, meat has been stuffed into natural casings, but nowadays alternatives, such as the co-extruded collagen casings are emerging. Co-extrusion technology uses a collagen dispersion which is applied directly on a stream of meat batter, whereby the casing is being formed at the moment the sausage is being produced. Currently, bovine hide split collagen is the primary source. However, due to rising costs, availability of natural casings, and increasing need for kosher/halal products collagen producers are searching for alternatives. Consequently, the manufacturers' interest in factors influencing extracted collagen quality is increased. This requires insight and understanding of the biochemistry of the collagen. Overall, the performance of obtained collagen is not only influenced by extraction, but environmental factors, such as animal's age, and breed as well.

The aim of the thesis was to provide fundamental knowledge for understanding the effects of biological factors on collagen quality of livestock animals for producing co-extruded sausage casing. This included three aspects: 1) investigating suitability of different animal species (poultry, cattle, pork); 2) investigating suitability of different collagen origins (skin, intestines, bladder); 3) investigating suitability of different breed/strain of animals. To obtain this knowledge, fundamental differences in collagen matrix structure were be investigated in relation to properties required in the co-extrusion process.

The **General Introduction** of this thesis starts with a description on several aspects of sausage casings followed by an overview of different casing types and casing functionality. Then continues with a description of the collagen structure, the co-extrusion process and bovine skin collagen. Subsequently alternative collagen sources as raw material for casing production in the co-extrusion process is described followed by factors influencing the collagen quality of co-extruded sausage casings and the associated knowledge gaps. Lastly, information is given about the approach investigated in this thesis, to understand the effect of biological factors on collagen quality of livestock animals for producing co-extruded casings.

Chapter 1 is a review focusing on the various aspects of bovine collagen dispersion used for co-extrusion production of high-quality sausages. The review starts with history of sausage making, followed by types of sausage casings and an explanation about co-extruded collagen casings focusing on bovine skin collagen, the production of colla-

gen from bovine skin, and the functional properties of co-extruded casings. Finally, the factors affecting collagen characteristics related to casing properties, potential other collagen sources and conclusion and future prospects are given.

Chapter 2 of this thesis describes the properties of different poultry skin sources in relation to co-extruded sausage casings. Results of this study showed that SDS-PAGE, rheology, DSC and TNBS showed little differences in parameters between the different chicken types. However, since film forming capacity was insufficient for broiler breeder and laying hen skins, it can be concluded that chicken skin from slower and fast-growing broilers were potentially suitable collagen sources for the co-extrusion process. In fact, it was possible with the dispersions prepared from these skins to create a strong film after salt precipitation, and an important property of a casing is its strength, as it dictates the initial sensory perception of a sausage.

Chapter 3 of this thesis describes effects of broiler weight and strain on skin collagen characteristics and their applicability for co-extruded sausage casings. The previous research has shown that particularly young broiler skins were suitable as a source for collagen gel for co-extrusion. This study focused on the effect of two other important factors, such as strain (slow and fast-growing) and body weight on the collagen properties. Results of this study showed minor differences in the biochemical and physical properties of the collagen dispersions. It can be concluded that based on the biochemical and physical properties, which were related to the final application, all four chicken collagen dispersions investigated have the potential of being a suitable collagen source for co-extrusion sausage casings.

Chapter 4 of the thesis evaluates the suitability of cattle skin collagen for producing co-extrusion sausage casing. Results of this study showed differences in complex viscosity, film forming capacity of the dispersions and mechanical strength of the films, with the skin of old cows appearing to be less suitable as a collagen source. A positive relationship between soluble collagen content and dynamic consistency index k^* was found. Furthermore, a negative relationship between soluble collagen content and Young's modulus of the films was found. It can be concluded that from the four investigated skins (American calf, Dutch heavy veal, Danish ox/heifer and German heavy cow), the skins of heavy cows were not suitable as an alternative collagen source.

Chapter 5 of the thesis evaluates collagen dispersions prepared from porcine urinary bladder and intestine as raw material for natural co-extruded sausage casings. Results of this study showed the potential of the application of porcine urinary bladder and intestines as an alternative collagen source for dispersion production for co-extrusion sausage casings. Higher shear force values were found for sausages produced with these

new raw materials compared to sausages produced with commercial bovine skin collagen. Moreover, improved secondary cooking properties were obtained with casings produced from urinary bladder and intestines. It can be concluded that porcine urinary bladder and intestine have great potential as raw materials for dispersion production for co-extrusion sausage casings.

The **General Discussion** of this thesis discusses how the biological factors influence the collagen quality of livestock animals for producing co-extruded casings, thereby discussing variables, such as extrudability, films strength, thermal stability that are important for the co-extrusion process. To improve the understanding on the effect of the biological factors on the collagen quality. This chapter ends with relationship in studied variables, availability of alternative collagen sources, main conclusions and research opportunities.

The main conclusion of this thesis are:

All investigated species (poultry, cattle, pig) and different collagen sources (besides cattle skin) appears to be suitable to produce dispersions for co-extrusion sausage casings. The older the animal, the less suitable the sources are to produce dispersions and co-extruded sausage casings.

The key factor appeared to be the pre-processing conditions for obtaining good quality collagen dispersions and films. Different sources need to be treated differently to achieve the best suitability.

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About the author

Patricia Suurs was born on December 21, 1977 in Wageningen. In 1997 she obtained her VWO diploma at the Hendrik Pierson College in Zetten, after which she started with the bachelor study Food Technology at the higher agricultural school (HAS, Den Bosch, The Netherlands). During the 3rd year of her studies, Patricia did an internship at the University of Lincolnshire and Humberside (Grimsby, United Kingdom) and worked on setting up a method for the analysis of polycyclic hydrocarbons in smoked salmon using High Performance Liquid Chromatography (HPLC). After successful completion of this study, Patricia obtained her Bachelor of Applied Science in 2001. Subsequently Patricia started her master study Food Science and Technology at the Wageningen University and Research specializing in product development and food chemistry. In 2003 she obtained her Master of applied Science. After this, Patricia started her job as an R&D technologist in February 2004 at Ruitenberg Ingredients B.V. in Twello. In this position, Patricia was involved in studying the physical and chemical properties of ingredients for the meat, fish and convenience industries. The focus was on the application of hydrocolloids and biopolymers for the meat industry. Measuring the rheological properties of raw materials and end products and developing measurement methods for quality purposes were part of the job. In October 2007, Patricia moved to Marel (formerly Stork Titan) to work as a research technologist, where she still works today. In this position, her focus is on generating knowledge in the field of carbohydrate and protein chemistry in relation to processes to produce sausage casings with co-extrusion equipment. She is engaged in gathering scientific knowledge, studying theoretical models, setting up and conducting experiments and translating the generated knowledge into technological principles for prototypes. The knowledge generated is used for the development of new equipment or processes for the processing of animal proteins. It is also her task to transfer the generated knowledge to the development department of Marel by giving internal presentations and writing reports. Patricia also represents Marel at (inter) national symposia and supervises students during their internship at Marel. Her work at Marel has resulted in several patent applications in the field of sausage production processes. In January 2019, Patricia started her part-time PhD trajectory at the adaptation physiology chair group at Wageningen University & Research, while continuing to support Marel at the same time. The results of this trajectory are described in this thesis and several chapters are published in international journals.

List of publications

Suurs, P. and Barbut, S., 2020. Collagen use for co-extruded sausage casings – A review. Trends in Food Science and Technology, 102, 91 – 101.
DOI: https://doi.org/ 10.1016/j.tifs.2020.06.011

Suurs, P., van den Brand, H., Daamen, W.F., Barbut, S., 2022. Properties of different poultry skin sources in relation to co-extruded sausage casings. Food Hydrocolloids, 125, 107434.
DOI: https://doi.org/10.1016/j.foodhyd.2021.107434

Suurs, P., van den Brand, H., Farawu, K., Daamen, W.F., Barbut, S., 2023. Effects of broiler weight and strain on skin collagen characteristics and their applicability for co-extruded sausage casings. Journal of Food Structure, 35, 100305.
DOI: https://doi.org/10.1016/j.foostr.2022.100305

Suurs, P., van den Brand, H., ten Have, R., Daamen, W.F., Barbut, S., 2023. Evaluation of cattle skin collagen for producing co-extrusion sausage casing. Food Hydrocolloids, 140, 108595.
DOI: https://doi.org/ 10.1016/j.foodhyd.2023.108595

Suurs, P., van den Brand, H., Daamen, W.F., Barbut, S., 2023. Collagen dispersions prepared from porcine urinary bladder and intestine as raw material for natural co-extruded sausage casings. *In preparation*

Training and supervision plan

Basic courses

WIAS Introduction Day	2019
Ethics and Animal Sciences	2021
Scientific Integrity	2022
Introduction to personal effectiveness	2019

Disciplinary courses

Writing Research Proposal	2019
Design of Experiments	2021
Introduction to R and R studio	2021
Food Proteins: functionality, modifications, and analysis	2020
Advanced Food Analysis	2022

Professional competences

Searching and organising literature	2019
Scientific writing	2021
Project and time management	2020
Supervising BSc & MSc thesis students	2021
Final touch: writing the general introduction and discussion	2022
Last stretch of the PhD programme	2022
Writing propositions for your PhD	2022

Societal relevance

Societal impact of your research	2021

Presentations and posters

ADP science day, Wageningen, The Netherlands	2019
7th Freiberg Collagen Symposium, Freiberg (virtual), Germany	2021
2nd ESM Conference, Wageningen, The Netherlands	2022
FCT-2022 Conference, Rome, Italy	2022
ADP science day, Arnhem, The Netherlands	2022

Teaching and Supervision BSc & MSc thesis

Lecturer – Meat Science students:

"Advanced collagen casings in sausage production"	2019, 2021, 2022

Martine Aben:

Optimization of collagen extraction and characterization of collagen from chicken skins	2020

Kuda Farawu:

Investigating the effect of growth rate/weight on collagen structure and quality of broiler chicken skin and the applicability for co-extrusion gels	2022

Robin ten Have:

The effect of age on bovine hide collagen structure and quality and the applicability for co-extrusion gels	2022

Aletta Podlin:

Co-extrusion production of casings to help revolutionize the meat industry	2022

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