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Quantitative Histological Analysis of Bovine Small Intestines before and after Processing into Natural Sausage Casings

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ABSTRACT

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

Processed intestines from pigs, sheep, and cattle are used as edible natural casings for the production of sausages. Given the large variety in sausages, specific qualities and calibers of casings are required from all species mentioned (Table 1). Unlike sheep and hog casings, beef casings are normally removed prior to consumption of the sausages *(23),* as they are thicker, tougher (similar to tendon), and therefore practically unpalatable. However, as consumption cannot be excluded entirely, an estimate on the presence of tissues related to the bovine spongiform encephalopathy (BSE) exposure risk in beef casings is warranted. Based on available import data for 2005 *(9),* it can be estimated that the beef casings used in Europe for the production of sausages amount to approximately €87 million, with a volume of 68,000 metric tons.

Since BSE was first officially diagnosed in the United Kingdom in November 1986, drastic measures have been taken by the European Union authorities to ensure consumers' safety. Among others, beef casings produced from European bovine intestines were designated specified risk material, and were banned from human consumption completely in 1997 *(6, 7).* However, BSE infectivity has so far only been confirmed in the distal ileum and not in any part of the intestinal tract used for the production of beef casings *(2, 12, 24, 28).* This precautionary ban terminated the production of beef casings in Europe and allowed only imports into the European Union from countries with a negligible BSE risk *(5).*

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

MATERIALS AND METHODS

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

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

When postmortem inspection was completed, the intestines were separated from the stomachs and transferred to the gut room

TABLE 1. *Examples of sausages and their natural casings*[a]

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

[a] Source: www.casings.com.

for immediate processing according to standardized operating procedures. The intestines were hand pulled from the mesentery and cut off with a knife approximately 2 m proximal to the ileocecal junction, forming 80 sets of intestines comprising the duodenum and most of the jejunum. The remainder of the small intestines, comprising the entire ileum and a small part of the jejunum, were removed and incinerated.

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

To obtain samples representing cleaned casings, the remaining 40 sets were turned inside out (standard operating procedure) and transferred to a Stridh-type cleaning machine (Mecanica Primitiva, Ltda., Salto Grande, São Paulo, Brazil) for processing. The sets were fed manually (top down) into the cleaning machine, where an adjustable, grooved, rubber roller positioned at the top of the machine transports the intestines downward into the machine toward two cleaning rollers. These rollers (turning clockwise and counterclockwise) have stainless steel ridges or ribs that scrape and remove the outer tissue layers (originally the lumenal surface), mainly the mucous membrane comprising the epithelium, lamina propria, and lymphoid tissue. The submucosa, muscularis, and serosa remain to form the wall of the cleaned casing. Samples were taken after this processing step and denoted as cleaned casings. A different cleaning procedure for hog and sheep intestines is described by Koolmees and Houben *(15),* which includes the complete removal of serosa and muscularis layers, leaving only the intestinal submucosa as the cleaned casing.

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

To determine the weight reduction due to the cleaning process, a separate study was done using similar Brazilian Nelore cattle. Sets of intestines ($n = 50$) were randomly selected, measured, and weighed at the first and second sampling point.

Histology. Lymphoid tissue occurs irregularly along the length of the small intestine as isolated lymphoid nodules (lymphonoduli solitarii), but tend to be most prominent in the ileum. These aggregated lymphoid nodules (lymphonoduli aggregati) are known as Peyer's patches *(13)* and are anatomically located on the convex side of the intestine opposite to the mesenteric attachment *(17, 27).* Complete rings of small intestine were therefore prepared for histology after fixation was complete to ensure that if any Peyer's patches were to be present in the cleaned casing, they would be detected. The subsequent histological examination, image analysis, and statistical analysis were done according to the methods described by Koolmees et al. *(16),* with minor modifications. The fixated intestinal segments (horizontal cross-sections) were embedded in paraffin wax by standard methods *(4, 13),* and two 5-μm serial sections were cut and stained with either hematoxylin and eosin or Picrosirius red *(10).* In total, 800 sections were prepared for each staining method from the samples collected.

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

Image analysis quantifying remaining lymphoid and neural tissue. Image analysis was done according to standard procedures for morphological measurements *(22).* Digital images of sections of the bovine intestines were taken with a uEye digital color camera, type UI-1440-C, resolution 1,280 by 1,024 pixels (IDS Imaging Systems GmbH, Obersulm, Germany). Image analysis was carried out with a PC-based system equipped with the KS400 software, version 3.0 (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the total area of the intestinal tissue per section. The total area was measured (in square millimeters) without magnification to allow a full view of the intestinal sample.

For the image analysis of the remaining mucosa, Picrosirius red–stained sections from 5 of 10 available zones (zones 1, 3, 5, 8, and 10) were examined (magnification of ×25) from both fresh intestine and cleaned casing samples. Four squares (fields of view) per section were randomly selected and the area of viewed squares occupied by mucosa (in square micrometers) was determined.

For the image analysis of the remaining lymphoid tissue, hematoxylin and eosin–stained sections from all samples containing lymphoid tissue, either before or after cleaning, were examined (magnification of ×25). The total area occupied by lymphoid tissue per section was measured (in square micrometers). Results of all measurements on remaining mucosa and lymphoid tissue were subsequently converted into percentages of the total intestinal tissue area of the section.

An anti–neuron-specific enolase antibody (monoclonal; Dako, Glostrup, Denmark) was used to stain the plexi of Meissner and Auerbach ($n = 10$), based on the methods described by Tersteeg et al. *(25).* A section of brain tissue was used as a positive control. Sections were examined using a Zeiss light microscope (magnification of ×400) with a projection head and a calibrated graticule. The surface area (in square millimeters) of the plexuses was calculated from length and width measurements.

Statistical analysis. A linear mixed-effects model *(1)* was used with a binomial distribution for the presence for lymphoid tissue, with ''animal'' as the random effect to model the correla-

TABLE 2. *Thickness measurements of intestinal wall, before and after processing*

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

[a] 95% confidence interval for the mean.

tion between the repeated observations within an animal. The independent factors are *cleaning*; the *location* of the sample taken from the entire length of the small intestine; the *facility,* indicating the slaughterhouse; and *country,* representing local differences in cleaning technique and breed. The penalized quasi-likelihood method was used for the approximation.

A linear mixed-effects model *(20)* has been performed with a normal distribution for lymphoid area and animal as random effect to model the correlation between the repeated observations within an animal. The lymphoid area is the dependent variable and the independent grouping variables are the cleaning method, facility, and country. The random effect was assumed to have a normal distribution. A variance model was used that allows a different variance per cleaning method. The maximum likelihood method was used for estimating the parameter effects.

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

In all analyses, the model with the smallest Akaikes Information Criterion (AIC) was selected as the best model *(19).* To determine a *P* value for the factors, a likelihood ratio test was performed to compare the different models. The models were fitted using the statistical program R, version 2.2.1 *(21).* Where possible, the 95% confidence intervals are given for the raw data that provide information on the accuracy of the respective findings *(3),* but no corrections were made for repeated measurements. However, when using these data in the linear effects models, the corrections for repeated measurements were included.

RESULTS

Thickness measurements. Table 2 summarizes the results of thickness measurements of the fresh bovine intestinal wall and of the cleaned beef casing. The results reveal that during processing, a significant decrease (one-sided Student's *t* test: $P < 0.0001$) in thickness occurred. The standard deviation of fresh intestine thickness is high. This can be explained by the considerable differences in thickness within the intestine wall caused by the presence of valves of Kerckring (plicae circularis) *(11).*

Compared with sheep and hog casings, beef casings are much thicker since they are composed of three tissue layers (submucosa, muscularis, and serosa) rather than one (submucosa only). The muscularis and serosa are removed during the processing of sheep and hog casings *(18).*

TABLE 3. *Average weights and length of 50 sets of intestines before (fresh intestines) and after (cleaned casings) cleaning*

Avg length[a]	Avg wt (g)[b]	SD	Relative wt (g/m)[b]	SD
Fresh intestines	3,900 (3,658–4,142)	874	110.7 (105.6–115.8)	18.4
Cleaned casings	2,264 (2,099–2,429)	596	64.0 (60.4–67.6)	13

[a] 35 ± 4 m, $n = 50$.
[b] 95% confidence interval for the mean.

TABLE 4. *Mucosa tissue present as percentage of total thickness*

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

[a] 95% confidence interval for the mean.

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

Mucosa. In the qualitative analysis, samples were scored on the presence of mucosa, before and after cleaning. As expected, all samples from fresh intestines ($n = 400$) contained mucosa. However, all samples from cleaned casings ($n = 400$) also contained residual amounts of mucosa.

Regarding the quantitative analysis, the lowest AIC was found when only the independent factor *cleaning* was taken into account. This means that the other factors (location, facility, and country) did not contribute to the differences found between cleaned casings and fresh intestines. There was a significant ($P < 0.0001$) reduction of 90% in mucosa tissue present in cleaned casings compared with fresh intestine (Table 4).

Lymphoid tissue. In the qualitative analysis, samples from fresh intestines ($n = 400$) and cleaned casings ($n = 400$) were scored for the presence of lymphoid tissue. In contrast to the presence of mucosa in all samples, lymphoid tissue was only found to be present in 60 (15% positive score) of the 400 fresh intestinal samples and 40 (10% positive score) of the 400 cleaned casing samples (Table 5). There was a significant difference ($P < 0.05$, odds ratio of 1.60, 95% confidence interval of 1.01 to 2.53) in the num-

TABLE 5. *Number of samples with lymphoid tissue present*[a]

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

[a] Number of samples found positive in each facility.

TABLE 6. *Lymphoid tissue present as percentage of total thickness*

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

[a] 95% confidence interval for the mean.

ber of samples containing lymphoid tissue when comparing fresh intestines ($n = 60$) with cleaned casings ($n = 40$). As only cleaned casings will be used for sausage production, for risk assessment purposes, account should be taken of the 1:10 incidence ratio for the presence of lymphoid tissue.

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

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

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

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

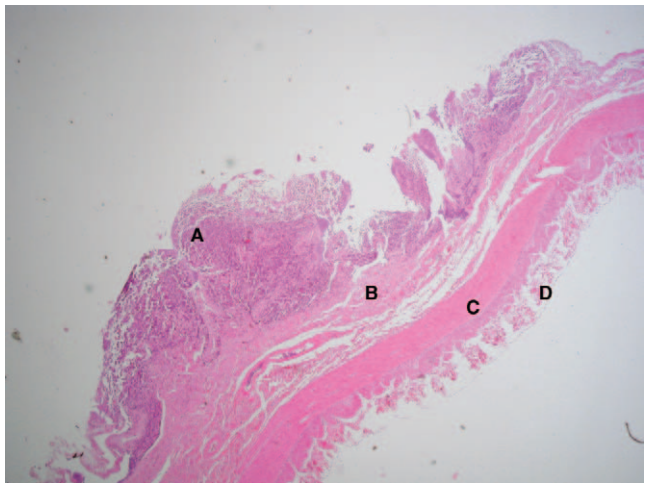

FIGURE 1. *Cleaned beef casing (hematoxylin and eosin, ×25 magnification) with remains of Peyer's patches embedded in mucosa (A), submucosa (B), muscular layers (C), and serosa (D).*

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

DISCUSSION

Several different cleaning procedures are in place within the casing industry for the processing of bovine intestines into beef casings. However, the differences in these procedures relate mostly to the level of automation, whereas the general principle of cleaning remains the same throughout. A pilot study was done by Koolmees in 1998 on the histology of beef casings *(14),* using a fully automated process (H+H automatic beef casing line, type 60-120RD4-5 Holdijk + Haamberg GmbH, Gronau, Germany). Preliminary results found in this study were compared with the current quantitative analyses, indicating a similar level of cleaning efficacy. Therefore, as all cleaning procedures are based on the same cleaning technique, and there was no *country* effect on the quantitative results found in the current study, it can be inferred that all cleaning procedures will lead to a similar quantitative reduction of mucosa and lymphoid tissue.

As shown, beef casings retain some mucosa and lymphoid tissue after the cleaning process, and all layers of the intestinal wall can be distinguished (Fig. 1). This also means that no neural tissue is removed, leaving the plexi of Meissner (embedded in the submucosa) and Auerbach (between the circular and longitudinal muscle layer) intact.

Previous reference to histological textbooks *(11, 17)* shows lymphoid tissue to occur irregularly along the length of the small intestine as isolated lymphoid nodules (lymphonoduli solitarii) or as Peyer's patches (lymphonoduli ag-

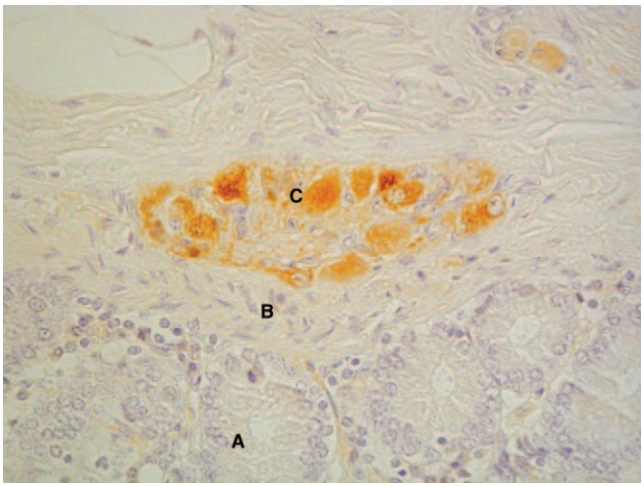

FIGURE 2. *Fresh intestine (anti–neuron-specific enolase antibody, counterstaining with Mayer's hematoxylin, ×400 magnification). Mucosal villi (A), submucosa (B), and plexus of Meissner (C).*

gregati). However, results indicate that due to the cleaning process, which removes Peyer's patches completely or partially, a more homogenous distribution pattern of lymphoid tissue occurs, based on these remnants and remaining isolated lymphoid nodules. These findings are substantiated by the fact that in the quantitative analysis of lymphoid tissue, the location from where the sample was taken along the entire length of the small intestine was without influence on the results. This is of great importance, as it will allow for a 1:10 incidence ratio on the presence of lymphoid tissue in a subsequent risk assessment, which can only be assumed when a homogenous distribution exists.

The presence of lymphoid tissue and myenteric plexi (Fig. 2) in cleaned beef casings does not implicate an inherent risk in transferring BSE. Sheep casings are considered to have a negligible BSE infectivity risk as Peyer's patches are removed completely after cleaning. From the neural tissue, only the plexus of Meissner will remain, as it is part of the submucosa that constitutes sheep casings *(16).* Contrary to the pathogenesis in sheep and mice, BSE infectivity in cattle has, up to now, an exclusive intraneuronal spread from the intestinal tract to the central nervous system *(2, 12).* Only in some of the clinically affected, experimentally induced and naturally occurring cases of BSE could sparse immunostaining of neurons in the myenteric plexi and disease-specific prion protein be detected in the Peyer's patches of the distal ileum *(24).* In cattle incubating BSE, disease-specific prion protein was only detected in the Peyer's patches of the distal ileum, not in the myenteric plexi of the distal ileum or elsewhere *(12).* Therefore, a negligible BSE risk can be assumed, as the distal ileum is removed completely prior to the processing of bovine intestines into beef casings, and the duodenum and jejunum are regarded as free of BSE *(2, 12, 24, 28).*

Using a similar argumentation based on existing literature, the U.S. Food Safety and Inspection Service (FSIS) amended in October 2005 its rule ''Prohibition of the Use of Specified Risk Materials for Human Food and Requirements for the Disposition of Non-Ambulatory Cattle'' *(26).* This amendment re-allowed the use of beef casings for the production of sausages originating from countries with a GBR II categorization, indicating a controlled BSE risk according to World Organization for Animal Health standards *(29).* A critical point in the FSIS amendment and World Organization for Animal Health Terrestrial Animal Health Code is the complete removal of the distal ileum from bovine intestines and prohibited use in the preparation of foodstuffs. This requirement is already fulfilled, as the ileum is unfit for casing production due to an aberrant shape and texture. It is therefore a standard operating procedure to remove and destroy the ileum prior to the processing of bovine intestines into beef casings *(8).*

TABLE 7. *Calculated weights of the different layers of cleaned casings*

Lymphoid tissue present	Area %	Approx wt of layers (g/m)
Mucosa	4.39	2.81
Lymphoid tissue	0.52	0.34
Neural tissue	0.14	0.09
Combined layer[a]	94.95	60.76
Total	100	64

[a] Consisting of submucosa, muscularis, and serosa.

Results indicate that approximately 50% of the total weight and thickness of the intestine was removed during the cleaning process (Tables 2 and 3). Mucosa is removed for 90% but remains present in all samples tested. Lymphoid tissue was either not present in 85% of the samples from fresh intestines or was removed completely in 90% of the samples from cleaned casings. In the samples from cleaned casings, 5.24% of total lymphoid area remained as percentage of the total thickness of the cleaned casing (Table 6). Assuming that the relative contribution of each specific tissue layer to the total weight is equal, a direct correlation is possible between the thicknesses of the relevant layers of the small intestine and their respective mass (Table 7). Taking into account the 1:10 incidence ratio of lymphoid tissue being present, it leads to a calculated area percentage of 0.52 for lymphoid tissue. Based on these data, it can be estimated that 340 mg of lymphoid tissue and 90 mg of neural tissue remain per meter of cleaned casing.

From an average length of 35 m of processed beef casings, 30 m is used for sausage production, with a stuffing capacity of 25 kg/30 m. Sixty sausages can be produced from each cut, as one standard sausage made in beef casings measures 50 cm and weighs approximately 400 g. If a meal consisted of half a sausage (200 g), then the casing envelope would be 25 cm long *(8).* According to the presented data, consumption of the entire 25 cm would result in the ingestions of approximately 0.09 g of lymphoid tissue and 0.02 g of neural tissue.

The results from this study clearly illustrate the limited presence of lymphoid tissue in cleaned beef casings, by incidence and calculated amount. As BSE infectivity remains unconfirmed in both lymphoid and neural tissue pres-

ent in the intestinal tract used as beef casings and the final exposure to the consumer is extremely limited, it can be suggested that beef casings carry a negligible risk in transferring BSE. Additional research using beef casings originating from BSE-infected cattle could further corroborate these findings.

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