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GERMFREE MUSCLE TECHNIQUES AS A TOOL FOR STUDYING MICROBIOLOGICAL ROLES IN SAUSAGE PRODUCTION.

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Sterile, undenatured tissue gives the research worker a valuable tool for the study of the effects of microorganisms on post-mortem tissue and to separate these effects from those caused by chemical and enzymatic reactions. Inoculation of this tissue, with specific microorganisms, also allows the measurement of the effect of the inoculation. This paper is a summary of the germfree work conducted at The Ohio State University during the last 10 years.

Five techniques have been developed and utilized at OSU to obtain germfree tissue, or tissues with extremely low levels of microorganisms. These procedures are gnotobiotic animals, surgical isolator, V-trap, flaming, and core techniques.

METHODS FOR OBTAINING STERILE, UNDENATURED TISSUE

Gnotobiotic Method

Gnotobiotic animals were taken from the pregnant female by cesarean section and grown with germfree procedures. The essential equipment was steam or heat sterilized and sterile air-locked into the isolators where the animals were exsanguinated, eviscerated, cleaned, processed and packaged in containers within the isolator. Gases for packaging were introduced through the inlet filter. A vacuum was obtained by attaching a glass tube containing a cotton filter to the package and connecting this to a vacuum source after the package was removed from the isolator. Self-sealing, flange-type, rubber stoppers were incorporated into the package when inoculations were necessary.

Surgical Isolator Method

An animal grown under normal environmental conditions was slaughtered using surgical sanitation during the sticking operation. The hide, with the hair removed, remained on the carcass in the area where the sample was to be withdrawn. A sterile surgical isolator, connected to a processing isolator was attached to the hide with double adhesive tape (Figure 1). The incision was made through the isolator floor and hide followed by sterilization with an electric cautery. Sterile samples were removed, transferred to the processing isolator, and handled identical to those of the gnotobiotic animal.

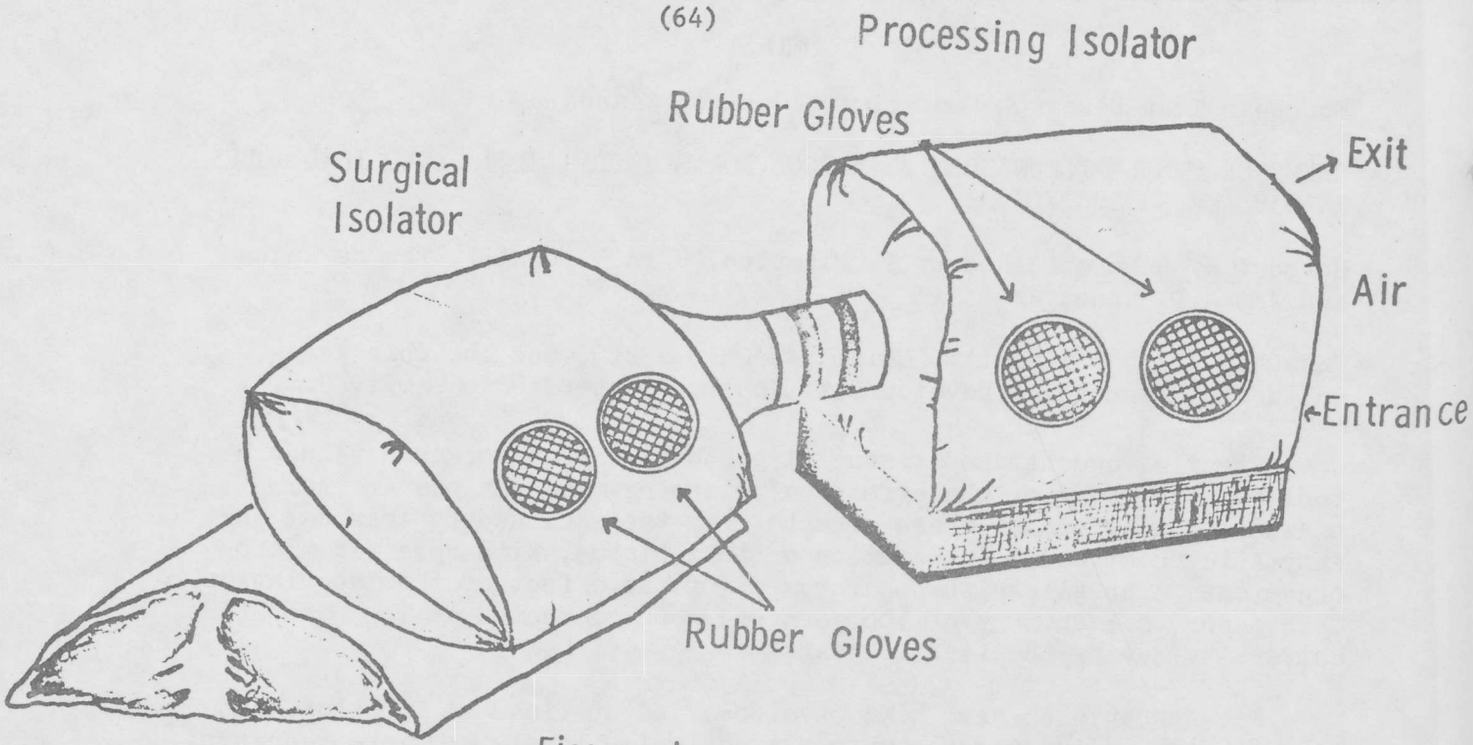

Figure 1. Surgical Isolator Technique.

V-Trap Method

This technique consisted of submerging a conventional tissue sample into a V-trap filled with hot water and transferring the sample out the other end of the trap into a sterile isolator (Figure 2). The denatured external surface was trimmed away within the isolator and discarded. The internal undenatured tissue was then used as the low microbial level sample. Processing and packaging were completed as previously mentioned.

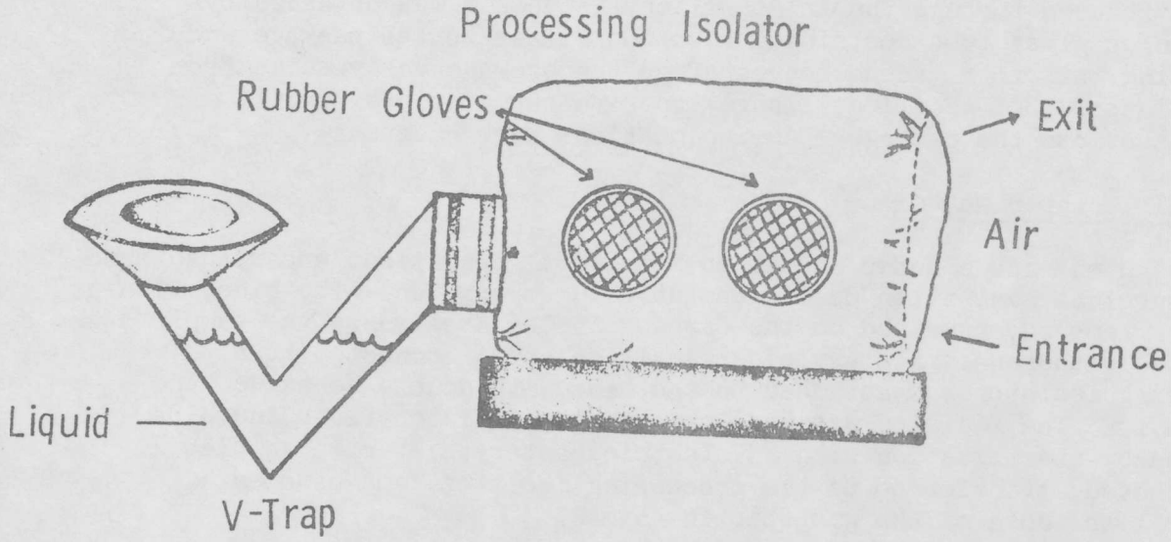

Figure 2. V-Trap Isolator Technique

Flaming

Poultry grown under normal environmental conditions was slaughtered in a sanitary manner and dry picked. The carcasses were bathed in alcohol and flamed. They were then placed into a sterile isolator equipped with ultra-violet lights. The external denatured tissue was removed and the internal undenatured tissue was used as a sample. Packaging was conducted in the previously described manner.

Core Method

A sampling device was made by attaching a metal coring tool to the mouth of a sample container. A paper cap was placed over the end of the tool and the entire unit sterilized. After surface trimming with a sterile saw and/or knife, germfree tissue was collected by inserting the sterile coring tool into the fresh surface followed by withdrawal and subsequent deposition of the sample with the aid of a sterile plunger into the sample container (Figure 3). The coring tool was then detached from the sample container and the container sealed.

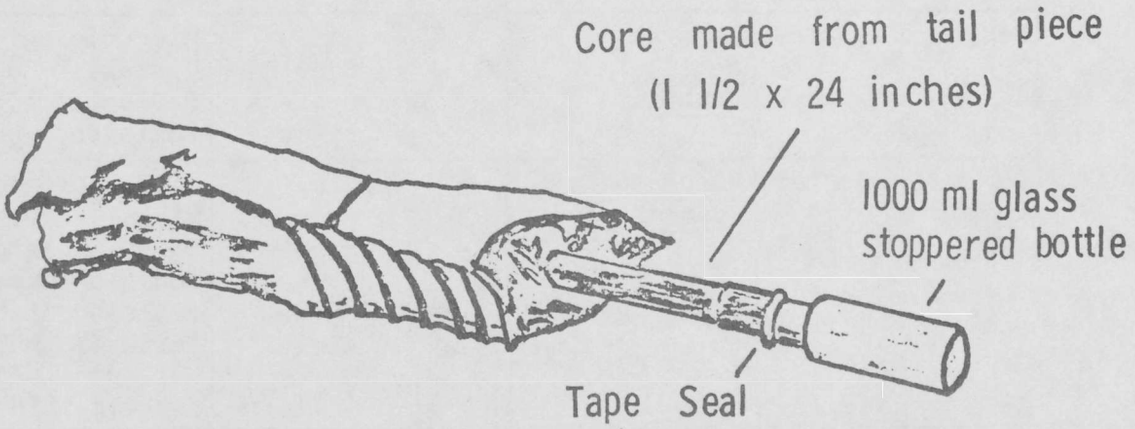

Figure 3. Core Sampling Technique.

Muscle tissue was usually sterile at the time of slaughter. The gnotobiotic animal and the surgical isolator techniques were successful in producing sterile samples in a large percentage of the trials. The V-trap, flaming and core techniques were successful in providing sterile or low microbial load tissue samples.

Table 1 is a summary of the sterile tissue work conducted at OSU during the last 10 years.

Table 1. Sterile Tissue Work Conducted at OSU

Techniques for obtaining low microbial level tissue	Species used	Tissue studied	Inoculations used	Storage Temperature	Environment & treatment	Evaluation made
Gnotobiotic Animals	Swine Rabbits Chicken	muscle, liver, lungs, spleen, heart, kidney	none	-6°C, 10°C, 23°C, 37°C, 100°C	Air, N_2, O_2, vacuum	Microbiological, Exudate, Visual, Odor, Microscopic
Surgical Isolator	Beef	muscle	general, general (low level), Pseudomonas, Achromobacter	3°C	Air	Microbiological, Protein Fractionation, Water holding capacity, pH
Dip (V-Trap)	Rabbit	muscle	general	3°C	Air	Microbiological, TBA Values, Visual, Odor, pH
	Beef	muscle	general, Bacillus subtilis	3°C	Air, Pre- & post-rigor	Microbiological, Visual, Tenderness, Reflectance, Emulsion capacity
	Pork	muscle	Pseudomonas putrefaciens	3°C, 23°C	Air, Pre- vs. post-rigor, Emulsion products	Microbiological, Visual, ERV, Odor, Exudate, pH Emulsion capacity, Emulsion stability, Texture, Emulsion: flavor, odor, shelf-life
Flame	Turkey	muscle	Pseudomonas fluorescens, mixture of Flavobacterium & Alcaligenes	-5°C, 20°C	Air, Cooked vs. Uncooked	Microbiological, Weight loss, Visual, Odor, Exudate
	Chicken	muscle	Pseudomonas putrefaciens, Flavobacterium, Alcaligenes	3°C	Air	Microbiological, pH, Flavor, Fatty Acid analysis
Core	Pork Beef	muscle	Achromobacter, Escherichia coli, Pseudomonas putrefaciens, Bacillus subtilis, Leuconostic mesenteroides, Lactobacillus casei	3°C	Air, Microwave cooking, Nitrite, Nitrate	Microbiological, Visual, Cooking losses, Taste, Nitrate reduction

RESULTS

Visual and Odor Observations

Visual color of mouse muscle tissue was altered only slightly during several months when stored at 23°C under vacuum and nitrogen. The appearance of liver tissue deteriorated more rapidly than corresponding muscle tissue obtained and stored under similar conditions. Heart, lung and spleen became darker with age. Visual deterioration was accelerated for all tissue, when storage temperature was increased and when greater amounts of oxygen were added to the atmosphere. After being stored for a month at 5°C, rabbit and pork muscle changed only slightly in visual appearance. Turkey tissue maintained a good appearance for one year at 5°C. In all cases, inoculated tissue deteriorated most rapidly in visual color.

Reflectance evaluation (685 m μ) of uninoculated beef tissue, showed an increase in reflectance (lighter color) during 14 days storage at 3°C. The samples obtained post-rigor, had higher values than the samples excised pre-rigor. The inoculated samples followed these same trends, but in all cases, the reflectance of these inoculated samples was lower (darker) than for the comparable uninoculated samples.

Sterile pork muscle tissue showed little deterioration in texture during 3 days of storage at 23°C compared to the less firm texture observed in the corresponding inoculated tissue. Some exudate has been observed in most species during sterile storage and white crystals have been found on the surface of samples after prolonged storage.

No off-odor was detected from sterile poultry tissue stored for up to 1 year at 3°C. With increasing temperature, a slightly stale to musty odor was observed in rabbit tissue. There was no indication of a sour or pungent odor which was evident in inoculated tissue.

In summary, it may be observed that visual appearance and odor were not altered drastically when sterile tissue was stored at room temperature or below.

Microscopic Evaluation of Sterile Tissue

A loss in quantity of stained nuclear material and a degradation of the muscle fiber was noticed for muscle tissue stored 32 days at 23°C. It would appear that the micro structure of sterile tissue was altered more than was obvious from the macro visual appearance.

pH

The pH dropped normally during rigor for chicken, pork and beef and the rigor pH was maintained at approximately this level, until altered by microorganisms. *Flavobacterium*, *Alcaligenes*, *Pseudomonas putrefaciens*, *Achromobacter*, and *Bacillus subtilis* caused an increase in inoculated muscle pH and *Lactobacillus casei* caused a decrease in muscle pH. An example of the pH pattern may be seen in Figure 4.

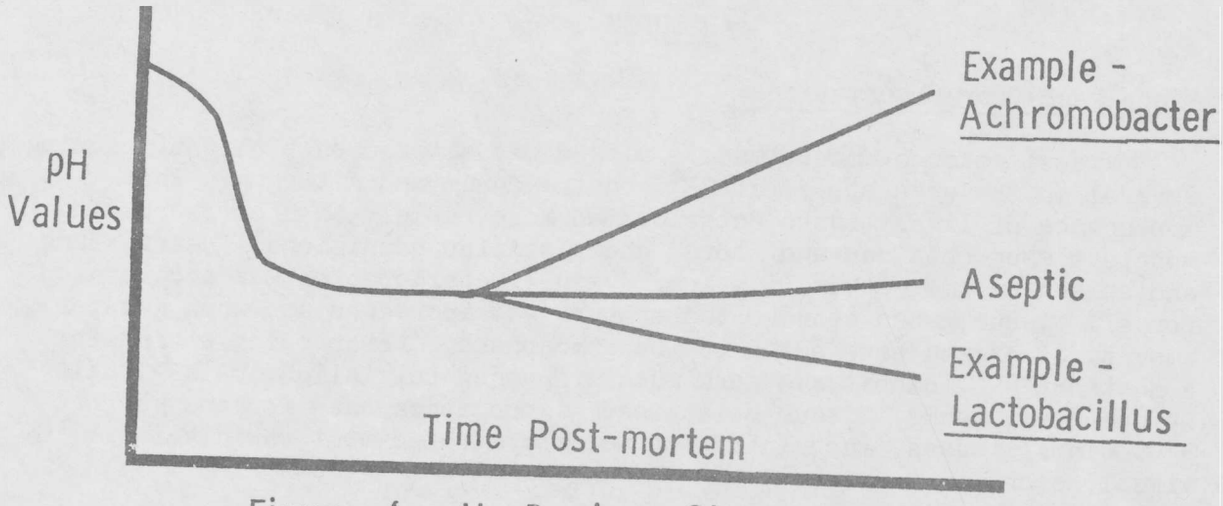

Figure 4. pH During Storage

No obvious difference in initial pH decline was observed when comparing sterile tissue with pre-rigor inoculated tissue.

It would seem that the major pH alteration pre-rigor was due to enzymatic and/or chemical reactions of the tissue. The major alteration (either increase or decrease) post-rigor was due to the growth of micro-organisms in the sample.

Protein Fractionation

Sterile and comparable inoculated tissue (inoculated with micro-organisms that raise pH) have been evaluated during post-mortem storage ($3^{\circ}C$). Sarcoplasmic soluble protein decreased during storage for both the sterile and inoculated tissue; however, the decline was much slower for inoculated tissue. The myofibrillar fraction increased slightly for 10 days and then remained relatively constant for the next 25 days in sterile tissue. The quantity of myofibrillar fraction rose significantly faster for the inoculated tissue during the first 10 days and remained above the sterile fraction. During aseptic storage a slight increase in stroma protein was noted. A slight, but significant, decrease in stroma protein was noted for inoculated tissue. Only a slight increase was noted in the non-protein nitrogen fraction during sterile storage, but a large and significant increase was observed in the comparable inoculated tissue.

When storing the sterile tissue for 35 days, the sarcoplasmic fraction decreased whereas the myofibrillar and non-protein nitrogen increased. Storage of inoculated (with microorganisms that raise pH) tissue revealed a decrease in the sarcoplasmic and stroma fractions and an increase in the myofibrillar and NPN fractions. This would indicate that protein solubility is altered to a major degree by chemical and/or enzymatic reactions and can also be significantly influenced by the growth of micro-organisms.

Fatty Acid Analysis

The fatty acid composition of poultry tissue was evaluated after 14 days storage at 3°C. A significant decrease was observed in oleic acid for tissue inoculated with Alcaligenes and Pseudomonas putrefaciens. This suggested that oxidation of the fat was accelerated by these species of microorganisms. No significant change in fatty acids was noted for the Flavobacterium inoculum nor in the uninoculated tissue.

TBA Values (2-Thiobarbituric acid)

Rabbit tissue, obtained by the hot water dip procedure (V-trap), had an increase in TBA values when compared to the inoculated (general) tissue. This indicates that hot water, or peracetic acid (used to sterilize the isolator), increased oxidation. Upon storage (3°C for 38 days), both inoculated and uninoculated tissue increased in TBA values.

Water Holding Capacity

In sterile beef tissue, the percent free water increased during rigor and then remained relatively constant for 35 days storage at 3°C. In inoculated (using microorganisms that raise pH) tissue, the percent free water also increased during rigor but then decreased significantly during storage (Figure 5).

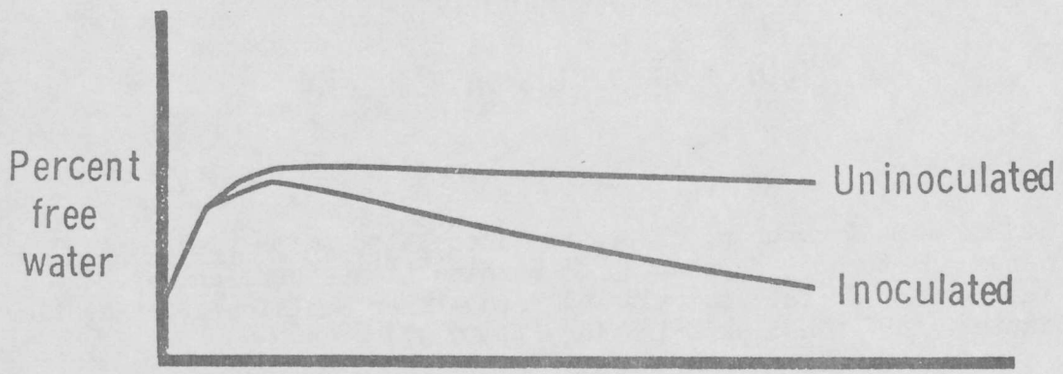

Figure 5. Water Binding

ERV (Extract Release Volume)

ERV values for porcine tissue rose during rigor for both uninoculated and inoculated tissue. Uninoculated tissue ERV values declined slightly during 9 days of storage. The ERV values for tissue inoculated with Bacillus subtilis and Lactobacillus casei decreased at a more rapid rate and tissue inoculated with a general inoculation (caused an increase in pH) decreased at a much more accelerated rate.

Both water holding capacity and ERV values are influenced by pH alterations. It would appear that the pre-rigor alterations in these two measurements were influenced by chemical and enzymatic reactions and that major alterations post-rigor were due to microbial growth with the pH achieved during growth being very important.

Emulsifying Capacity

Emulsification capacity, of uninoculated beef tissue, decreased during the first 4 days post-mortem and increased slightly during 30 days storage at 3°C. In a general inoculated (microorganism raised pH) tissue, the increase in emulsifying capacity occurred earlier and was greater (Figure 6).

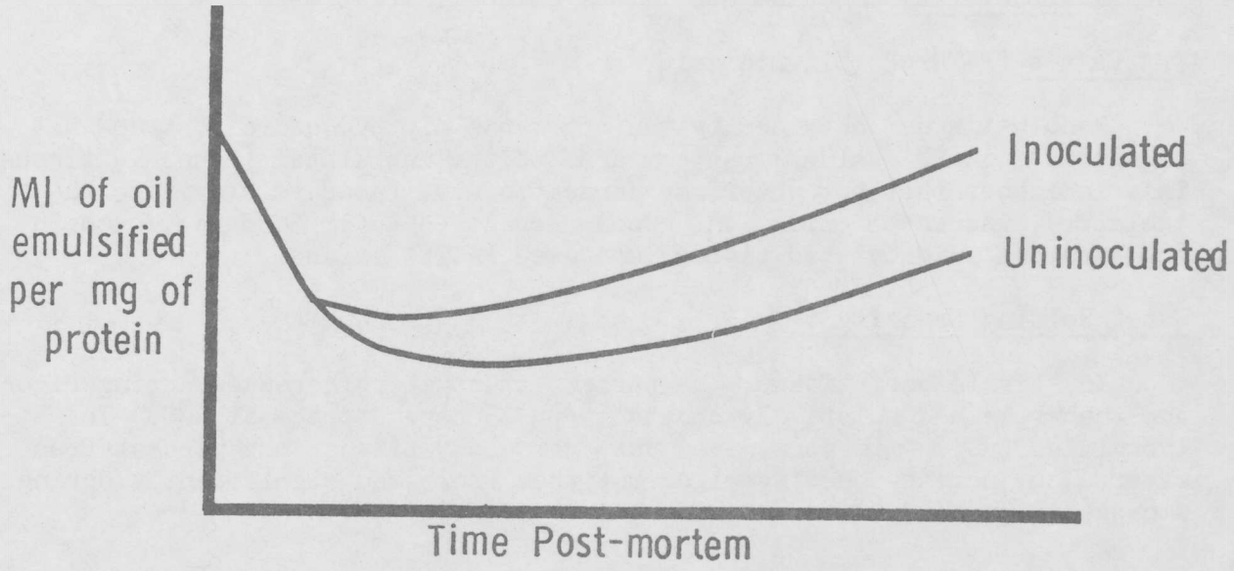

Figure 6. Emulsion Capacity

Emulsion Stability

Emulsions made from uninoculated and inoculated (with Pseudomonas putrefaciens and stored 3 days at 23°C) porcine tissue were compared at the 30% fat level. No fat was released from either emulsion after heating for 15 minutes at 90°C and centrifuging (1,000 G).

Sausage Manufacture

Taste panel results from a manufactured emulsion product made, from inoculated, and non-inoculated porcine comminuted product may be seen in Table 2.

Table 2. Taste Panel Evaluation of a Bologna-Type Product.[a/]

	Non-inoculated Tissue Bologna	Inoculated Tissue Bologna
Flavor	7.22	4.50
Off-flavor	9.75	8.00
Juiciness	7.08	5.83
Texture	7.00	5.50
Firmness	7.88	5.45
Color	4.58	4.50

[a/] Hedonic scale: 10 = excellent; 1 = unacceptable

For all characteristics evaluated, the non-inoculated comminuted product was rated higher than the comparable inoculated product, with the largest difference being in the flavor category. The lack of flavor appeared to be a greater problem than objectionable flavor for inoculated products.

Addition of nitrite to sterile uncooked tissue resulted in a normal cured color development, but the addition of nitrate did not develop a cured color even after 14 days of storage at 3°C.

Cooked Tissue

After subjecting sterile poultry tissue to cooking, the microbial growth rate on such tissue was more rapid than with comparable uncooked sterile tissue. When poultry tissue was subjected to a taste panel, the panel preferred: fresh tissue, uninoculated tissue and inoculated tissue, in that order. In gas chromatography analysis of the head space of stored poultry products, 35 peaks were obtained. After 1 year storage at 5°C, sterile poultry tissue still appeared fresh in color when compared to sterile cooked tissue, which was pale in color. Warner-Bratzler evaluation for tenderness indicated that sterile beef samples, removed pre-rigor, decreased in tenderness during rigor and then increased during the remainder of the 14-day storage period. Inoculated samples increased in tenderness at a more rapid rate than uninoculated samples during post-rigor storage. Sterile samples taken from the beef carcass post-rigor, maintained a relatively uniform tenderness for hogs which was more tender than the samples taken pre-rigor. Tenderness then increased during the next 7 days of storage. In this case, inoculated samples also increased in tenderness more rapidly than uninoculated tissue.

SUMMARY

Five techniques were described for obtaining undenatured tissue samples, which have not been contaminated with microorganisms, or contaminated only with low levels of microorganisms. These samples allow the separation of tissue changed into those caused by microorganisms and those caused by chemical or enzymatic reactions. Most reactions, which occur pre-rigor, are caused by chemical and enzymatic reactions. Those which occur post-rigor are a result of microorganisms, chemical and enzymatic reactions. At elevated temperatures and at a higher oxygen content, in the storage environment, the influence of microorganisms was accelerated. The direction of the pH alteration, caused by the microbial flora, also influenced a great number of the physical parameters that are very important in sausage production.