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INFLUENCE OF A BIO-CONSORTIUM STARTER CULTURE ON THE BIOCHEMICAL AND MICRO STRUCTURAL CHARACTERISTICS OF TRADITIONAL DRY CURED MEAT PRODUCT

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Abstract

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In this paper, we focus on a comparative evaluation of the biochemical, micro structural and sensory characteristics of a traditional Bulgarian dry cured meat product – Elena fillet, made using a natural fermentation process under the effect of a bio-consortium starter culture from *Lactobacillus plantarum*, *Lactococcus lactis* and *Micrococcus varians*. The starter culture effect on the biochemical transformations in the protein fraction was determined by monitoring the changes in the degree of hydrolysis and the electrophoretic profiles of meat proteins. Transmission electron microscopy (TEM) was used for evaluation of the micro structural changes during fillet drying and ripening. Similar dynamics of the biochemical and micro structural transformations were observed in all samples, with and without a starter, but they were more pronounced in the inoculated products. In the latter, the histological changes related to microstructure improvement started much earlier (the 7^{th} – 14^{th} day), which coincided with the growth of the lactic acid bacteria (LAB) introduced. In these samples, under the effect of the starter culture used and the enzyme activity of meat proteases in the anisotropic (A) and isotropic (I) sectors, stronger destruction of the actin and myosin protofibrils in *m. Longissimus dorsi* occurred. The inoculated samples exhibited partial Z-line hydrolysis in the middle of the I-disk and emergence of elongated light strips filled with finely granulated matter.

This was related to acceleration of the ripening processes and a higher sensory evaluation of the ready-to-eat inoculated dry meat products.

Key words: dry cured meat, starter culture, microstructure

Introduction

Over the centuries, people learned through practice how to control the fermentation processes accompanying the production of dry cured sausages, which led to the formation of a group of fermented meat products with region-specific characteristics, highly prized and sought after for their unique sensory and nutritional properties. This group includes a large number of different products with varying recipes, drying and ripening periods, sizes and kinds of meat raw materials, taste and smell, texture and acidification rate (Talon et al., 2007). The different production factors such as specific meat batter composition and the production technology applied select a limited number of strains which are competitive enough to dominate the ripening and drying processes (Cocolin et al., 2009; Olesen and Stahnke, 2003; Olesen and Stahnke, 2004; Rebecchi et al., 1998).

Although the problem of using starter cultures in meat product technology has been studied since the 1950s – 1960s, it has gained new significance owing to the search for new functional starter cultures. They could offer one or more additional properties to complement conventional `starters by enhancing and optimizing the meat product fermentation process and by contributing to the production of tastier, safer and healthier products (Ammor and Mayo, 2007; Aymerich et. al., 2004; Leroy et. al., 2006).

Therefore, the choice of the most suitable starter culture for a particular kind of product would be of utmost impor-

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tance in order to preserve the specific sensory characteristics of traditional dry cured meat products and expand the market by introducing new kinds of products. An essential factor in the selection of a suitable starter culture is the evaluation of its adaptive capacity in the micro ecosystem of meat raw materials and its participation in the fermentation processes during the carbohydrate, protein and lipid transformation throughout ripening and drying.

This paper studies the effect of a bio-consortium starter culture on the biochemical, microbiological and micro structural changes with a view to improving the quality of Elena fillet – a traditional Bulgarian dry cured meat product.

Materials and Methods

Starter culture formulation and dry cured Elena fillet manufacture

Lactina Ltd. Bankya, Bulgaria, produced the bio-consortium starter culture. It was selected on the basis of the biochemical properties of strains in order to provide certain technological advantages, and was composed of a combination of the following bacterial species: *Lactobacillus plantarum:Micrococcus varians:Lactococcus lactis* = 1:1:1. The lyophilised starter culture contained at least 10^{12} live cells per gram.

For evaluating the effect of the above starter culture on the biochemical and micro structural characteristics of traditional Bulgarian dry cured Elena fillets, two samples were prepared: a test sample (MT2) to which the respective starter culture was added, and a naturally fermenting sample used as control (C).

The above products were prepared using skinned pork sides of the Bulgarian White Swine breed, with average live weight of 90 kg. After cooling at 0 – 4°C for 48 hours, *m. Longissimus dorsi* was separated. Following the removal of fat and fascia, the pork tenderloin was cut into 500 g pieces and salted using a saline solution in 16°Be concentration. The starter culture was added to the salting solution in an amount providing 10^6 cells/g raw meats. The salting was conducted at 4° ÷ 6°C for 48 hours. After salting, the test and control samples were drained in a drying chamber in two stages: at a temperature of 18 ± 1°C and 90 ÷ 85% relative humidity for 48 hours, and at a temperature of 15 ± 1°C and 85 ÷ 80% relative humidity for 24 hours. The drying was performed at 12 ÷ 14°C and relative humidity of 80 ÷ 75% for 16 days until the water content reached 550 $g.kg^{-1}$ meat product.

The effect of the bio-consortium starter culture on the microbiological, physicochemical, biochemical and microstructural changes in the Elena fillets studied was evaluated by performing tests on the raw material, then after salting and draining (on the 5th day), during drying (on the 7th and 14th day), and on the final products (on the 21st day). On the 21st day of their manufacture, the final products were rolled into a homogenised mixture of spices (savory and black pepper) and vacuum-packed. The storage proceeded under refrigeration conditions until the 28th day, when microbiological and physicochemical analyses were made again.

Microbiological analysis

Three replications for each sample were studied on the 5th, 7th, 14th, 21st and 49th day. The samples were prepared by transferring 25 g of each sample into a sterile stomacher bag, then adding 225 ml of diluent (physiological saline: 8 g/1L NaCl) to it, and homogenising it in a Stomacher for 2 minutes. The subsequent tenfold dilutions with the same diluent were prepared and the further analyses were performed with double replication for the following microbiological indices: the total number was enumerated on PCA after 48 h at 30ºC, the lactic acid bacteria were enumerated on MRS Agar (HiMedia) after 48 h at 37ºC, the Micrococcaceae – on Mannitol Salt Agar (MSA, HiMedia) after 48 h at 37ºC, the Enterococci – on Esculin Azide Agar (HiMedia) after 48 h at 37°C ; the coliforms on HiCrome Coliform Agar w/SLS (HiMedia). After enumeration, the mean values and standard deviation were calculated.

Physicochemical analysis

The pH values were measured potentiometrically in a water extract of the sample 1:9 (w/v). Three independent measurements were made for each sample. Water activity (a_w) was measured using a LabSwift-aw system (Novasina AG, Switzerland) at 25°C. To determine moisture, drying of a homogenous mixture of the samples at 104 ± 1°C was performed using an electronic moisture analyzer model KERN MLS-A (Kern & Sohn GmbH, Germany). The studies aimed at evaluation of the residual nitrite quantity were made using a M550 Double Beam Scanning UV/VIS spectrophotometer (Camspec Ltd, United Kingdom) according to BDS EN 12014-3. The colour characteristics of the cut surface of the tested samples were objectively determined using a Minolta ChromaMeter (model CR 410, Osaka, Japan) in the CIE Lab system, where L* was brightness, a* was the red colour component and b* was the yellow colour component. The measurement was conducted using illuminant C and 2° standard observation angle. All measurements were taken five times in non-overlapping zones, and the mean values and standard deviation were calculated.

Biochemical and microstructural analysis

The proteolysis index (PI) was calculated as the ratio between NPN and TN multiplied by 100 (Latorre-Moratalla et. al., 2010). TN and NPN contents were determined by the

Kjeldahl method (AOAC, 2005). The NPN fraction was previously extracted from the sample with 0.6 M perchloric acid (Diereck et. al., 1974).

Meat protein extraction was performed using a phosphate buffer with pH 7.3 and 0.55M ionic strength, and the protein concentration was determined according to the Lowry method as described by (Vasilev et. al., 2012). The proteins were analysed by SDS PAGE according to Laemmli (1970) on 12% polyacrylamide gel using the Cleaver (Scientific Ltd.,UK) electrophoresis equipment. The electrophoretograms were run with Precision Plus Protein Standards (Bio-Rad) of known molecular weight (10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kDa), and the gels were subsequently stained for 20 min in a 0.2% (w/v) solution of Coomassie brilliant blue R in ethanol:acetic acid (40:7) and then destained for 24 h in ethanol:acetic acid (10:7).

For the transmission electron microscopy (TEM) the samples were fixed in 5% glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.2), then post-fixation was applied in 2% osmium tetroxide 0.1 M Millonig's phosphate buffer (pH 7.2) for 2 hours. Dehydration followed in an ascending gradient of ethanol concentrations. After propylene oxide treatment, the material was included in durcupan, a resin of the water-soluble group. Ultrathin silver-colour cuts were stained with uranyl acetate and lead citrate according to Reynolds (1963). The observation was performed on a Jeol 1200 EX (Japan) transmission electron microscope at an accelerating voltage of 75 kV.

Sensory analysis

The sensory evaluation of the samples was made on the 21st day. Non-trained panellists were recruited from the staff and students of the University of Food Technologies, Plovdiv, Bulgaria, selected based on previous experience in consuming traditional dry cured Elena fillets. Furthermore, a preparatory session was held prior to testing, so that each panel could thoroughly discuss and clarify each attribute to be evaluated in fillets. The sensory analysis was performed along a hedonic scale. For each of the attributes evaluated, i.e. global appearance, colour, odour, ripened flavour, taste, sourness and texture, a scale of 1 to 5 was used, 1 being the dislike extremely, and 5 the like extremely evaluation of the respective attribute.

Statistical analysis

Two-way ANOVA analysis was performed to evaluate the effect of starter culture and time on physicochemical, biochemical and sensory characteristic. Fischer tests were performed to determine significant differences ($p \leq 0.05$). StatPlus 2009 software was used for statistical analysis.

Results and Discussion

Microbiological results

The results obtained on the dynamics in microorganism development during the manufacture (up to the 21st day) and storage (up to the 49th day) of the tested samples of Elena fillet dry cured meat products have been shown in (Figure 1). All tested dry cured fillets exhibited dominating presence of LAB and Micrococcaceae at the beginning of the salting and ripening process. In the MT2 samples, the LAB count was 2 log units higher compared to the control samples (C) as early as the 7th day. The LAB count in the inoculated samples continued to increase during ripening and drying and remained significantly higher in the final products compared to the control samples: 6.75 log cfu.g^{-1} and 4.9 log cfu.g^{-1} respectively. This tendency was also observed during the refrigerated storage of the samples under vacuum.

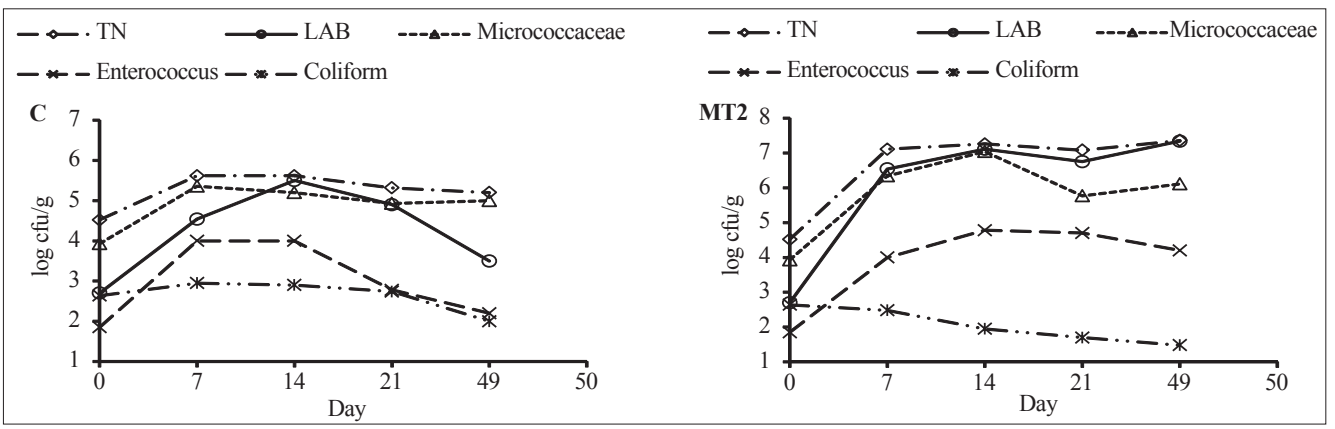

Fig. 1. Microbial growth during manufacturing process and storage of traditional Bulgarian dry cured Elena fillets: MT2 - test sample; C - control sample

The Micrococcaceae count in the control samples rose fast until the 7th day, and then did not change significantly during the drying and storage of the dry cured fillets. Other authors as well (Casaburi et. al., 2007; Kaban, 2013) have reported similar results about the dynamics in Micrococcaceae growth. In the MT2 samples, there was also a tendency towards an increase in the Micrococcaceae count until a maximum was reached on the 14th day (7.04 log cfu.g^{-1}), then a decrease was observed. Nevertheless, the Micrococcaceae count in the inoculated samples throughout the manufacturing process was 1–2 log units higher compared to the C samples.

The higher LAB and Micrococcaceae count in the inoculated samples could probably be attributed to the better adaptability of the selected strains included in the starter culture composition to the meat batter.

The initial enterococci count of 2 log cfu.g^{-1} rose in both the MT2 and the control samples until the 14th day, and then decreased. Higher values of the enterococci count in the inoculated samples (4.2 log cfu.g^{-1}) were observed on the 49th day. These results were inconsistent with the data reported by other authors (Casaburi et al., 2007). A possible reason for the higher enterococci count in the inoculated samples may be the fact that the *L. lactis* strain in the starter culture also forms a black precipitate upon Esculin Azide Agar, which hinders the correct enumeration of the enterococci.

With regard to the hygiene status, a similar tendency towards a decrease in the coliform count in the MT2 and C samples throughout the investigated period was observed. Nevertheless, the decrease in the coliform count was far more pronounced and reached a lower value in the samples containing a starter culture. This can be explained both by the lower pH and a_w values in the inoculated samples and by the capacity of the *L. plantarum* strain included in the starter culture composition to produce a large number of antimicrobial substances (Aymerich et al., 2000; Campanini et al., 1993; Dicks et al., 2004; Enan et al., 1996; Klingberg et al., 2006; Nowroozi et al., 2004).

Physicochemical results

The results on the changes in the pH, moisture content, water activity (a_w) and residual nitrite amounts have been presented in Table 1. No statistically significant difference was found in the water content and water activity values of the test and control samples of dry cured fillets ($p>0.05$). In both samples, the standard water content was reached on the 21st day of ripening and drying.

A similar tendency was observed in the pH changes in the test and control samples but there was a difference in the acid formation kinetics. In both sample types, pH initially decreased until the 14th day, reaching 5.64 in the control samples and 5.38 in the test samples. During the second period (14th – 21st day), the pH did not change in a statistically significant manner ($p>0.05$) in the samples prepared with and without a starter culture. During the refrigerated storage of the final, vacuum-packed products, the pH values increased but were not statistically distinguishable ($p>0.05$).

The statistical processing of the pH data showed that the starter culture introduced affected its changes ($p<0.05$). This confirmed the efficiency of the *L. plantarum* strain in initiating acidification, which has been reported by other authors as well (Hugas and Monfort, 1997).

The data on the residual nitrite amount and their statistical processing demonstrated that the starter culture used contributed greatly to the fast residual nitrite decrease ($p<0.05$). These data were in conformity with the higher Micrococcaceae count in the test samples and the nitrite reductase activity of the *Micrococcus varians* strain included in the starter

Table 1
The changes in the pH, moisture content, water activity (a_w) and residual nitrite amounts of control (C) and tested sample (MT2) during manufacturing process and storage

	Time, d						
	Sample	0	5	7	14	21	49
pH*	C	6.04	5.92	5.71.	5.64	5.69	6.08
	MT2		5.61	5.52	5.38	5.42	5.92
Moisture*, %	C	70.64	65.66	71.39	57.32	53.46	54.34
	MT2		61.18	67.13	55.32	54.88	53.81
Aw *	C	0.979	0.898	0.921	0.849	0.837	0.835
	MT2		0.862	0.904	0.834	0.846	0.823
NO^{2-} residue*, mg.kg^{-1}	C	ND	75.70	44.84	35.38	32.81	28.10
	MT2		58.73	23.23	22.34	20.27	16.53

*- values are the average of three measurements. ND – not determined.

culture as reported by other authors (Hammes, 2012; Selgas et al., 1988). A positive effect towards a faster nitrite reduction was also exerted by the pH decrease to close-to-optimum values for reactions of nitrite transformation to nitric oxide and nitrosomyoglobin formation (Honikel, 2008).

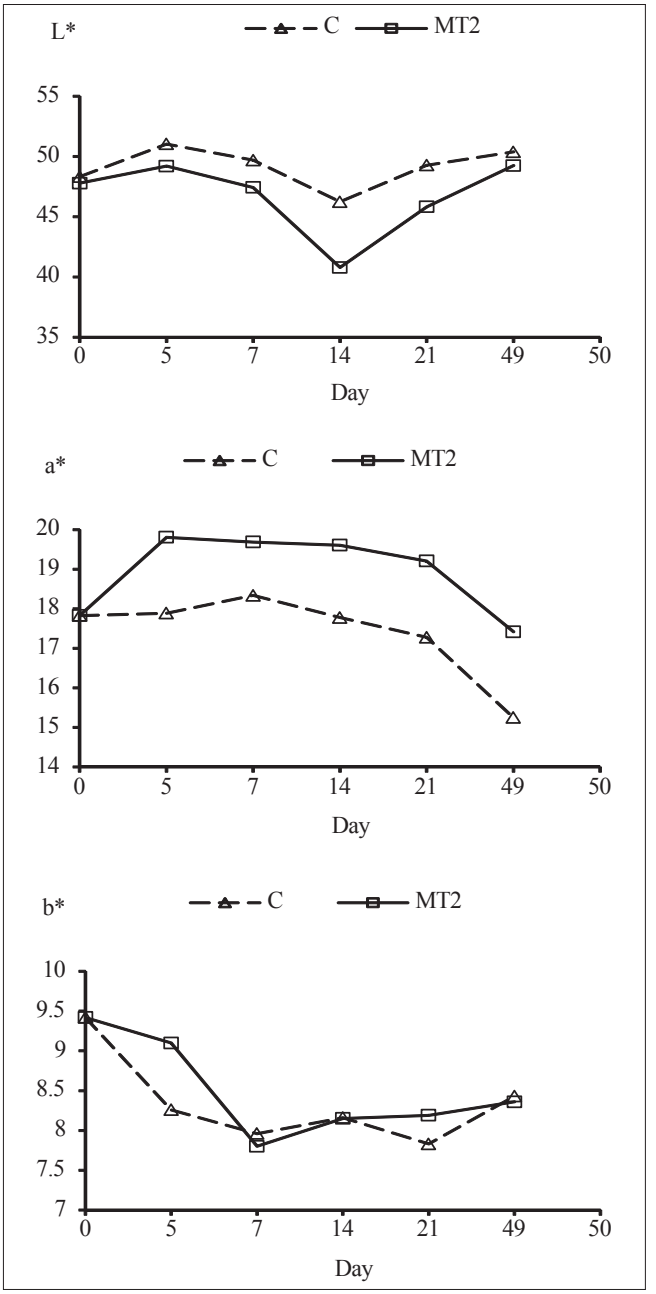

Fig. 2. Changes of the colour characteristics brightness (L*), red component (a*) and yellow component (b*) of control (C) and tested sample (MT2)

Colour characteristics

The data on the colour characteristics brightness (L*), red component (a*) and yellow component (b*) of colour have been presented in (Figure 2). The change in colour brightness L* in the test and control samples was similar in nature. There was a statistically significant difference in L* on the 14th day of the experiment, its values decreasing to 40.78 in the inoculated sample and 46.24 in the control sample. These data correlated well with the pH values of the samples and the ensuing changes in the meat protein structure. At the end of the drying period and the beginning of refrigerated storage of the final products, no statistically significant differences were established in the colour brightness of both sample types.

There was a different tendency in the changes of the red colour component (a*) values (Figure 2). In the final products (21st day), the a* mean value of the test samples was 19.21, as opposed to 17.28 for the control samples. The statistical differences between the red colour component values of the test and control samples corresponded to the results obtained on the dynamics of Micrococcaceae growth and nitrite reduction in the test and control samples. During the refrigerated storage of the vacuum-packed dry cured Elena fillets, a decrease in the a* values was observed, though they remained higher in the sample containing a starter culture.

Biochemical and Micro structural Results

Proteolysis index

The results on the proteolysis index (Figure 3) varied considerably until the 14th day of the manufacture of the samples; therefore, no clear tendency could be outlined. This coincid-

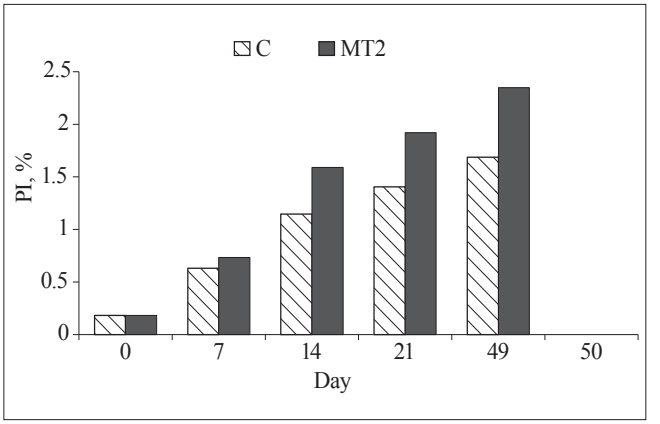

Fig. 3. Changes of the proteolysis index of control (C) and tested sample (MT2) during manufacturing process and storage of dry cured Elena fillets: MT2 - test sample; C - control sample, %

ed with the results reported by other authors (Latorre-Moratalla et al., 2010). Even so, after the 14th day of the drying and ripening of the test samples, a higher proteolysis index was established ($p < 0.05$), which testified to a deeper occurrence of hydrolytic changes, and was confirmed by electron microscopic analyses (Figure 5E). In the non-inoculated samples, PI increased at a much slower rate and remained below the values established in the inoculated samples throughout the manufacturing process.

Electrophoretic analyses

The SDS-PAGE profiles of the meat proteins of the finished products (21st day) have been shown in (Figure 4). The addition of a bio-consortium starter culture led to significant differences in the protein profiles. Extensive hydrolysis of the high molecular weight fractions, which corresponded to proteins with molecular weight of 100 – 250 kDa, occurred due to the decomposition of myosin (MHC) mainly. The actin fraction (43 kDa) underwent fairly poor hydrolysis during the ripening and drying of the MT2 samples. The intensity of the zone correspondent to the α-actinine (94 kDa) in the inoculated samples decreased in comparison with the control samples. This showed that the protein also underwent proteolytic changes during the ripening process. The inoculated samples exhibited an increase in the intensity of the zones between 50-70 kDa and between 25-37 kDa compared to the control samples. On the other hand, the myosin fraction in the control samples did not undergo any significant hydrolytic changes. Furthermore, the control samples contained protein fractions with molecular weight of 100-150 kDa, these being completely hydrolysed in the test samples. The results of the electrophoretic analyses confirmed the higher degree of proteolytic changes of meat proteins in the test samples under the combined action of endogenous enzymes and the proteolytic activity of the selected strains included in the starter culture composition.

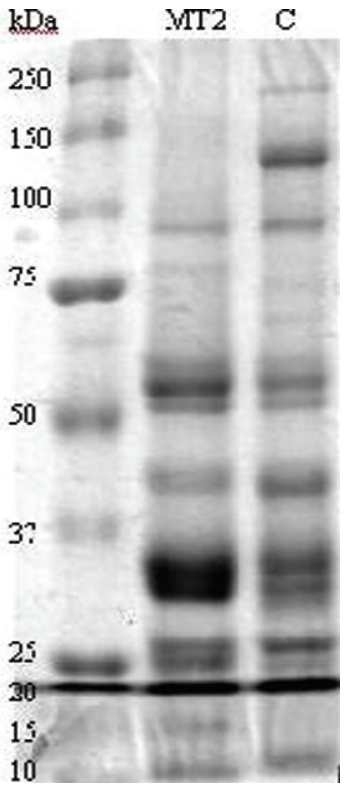

Fig. 4. SDS –PAGE patterns of protein hydrolysis in dry cured Elena fillets: MT2 - test sample; C - control sample on 21st day (final product)

Microstructural analyses

The electron microscope image of the muscle tissue (*m. Longissimus dorsi*) used as raw material for the preparation of the investigated samples of dry cured non-comminuted meat products of the traditional Bulgarian Elena fillet type showed a number of specific features (Figure 5). The longitudinal cut displayed the separate muscle fibres, with well outlined arrangement of myofibrils in straight lines and clearly visible sarcomeres of normal size. The Z-lines were well-preserved and delineated individual sarcomeres. In the histology diagrams of the investigated samples illustrated in Figure 5A, they can be seen as thick, dark lines in the middle of the isotropic (I) sectors. The anisotropic (A) sectors and H-zones were also well defined. Their location coincided in individual myofibrils whereby straight dark parallel lines were formed. A multitude of glycogen granules was visible in the sarcoplasmic space.

The results of the histological tests on the starter culture effect on the microstructure of the test and control samples of Elena fillet during the manufacturing process have been illustrated in Figure 5B, C, D, E. The histology diagrams of all investigated samples were characterised by three main features: 1) the muscle fibres were considerably modified compared to the raw material; 2) some of them were decomposed and partially hydrolysed in individual sections; and 3) there were intact muscle fibres situated in different sections of the block, unaffected by the enzyme activity.

It needs mentioning that the same regularity of the changes in the micro structural characteristics was observed in all investigated products but the changes were much more pronounced in the test samples compared to the control samples.

The results obtained from the control samples at the beginning of the technological process did not show any sig-

nificant qualitative micro structural deviations. During salting, especially in the manufacture of dry meat products from non-comminuted meat according to the classical technology, thickening of the structure occurred at first, because of the product dehydration, the increase in cooking salt concentration and related changes in meat proteins. The muscle fibres of the products contracted simultaneously with the structure thickening. The ultrastructural changes in the meat pieces indicated that during subsequent ripening and drying until the 14th day, as a result of partial protein hydrolysis, the Z-lines in the middle of the I-sectors in some sections were poorly fragmented but still the individual sarcomeres were outlined (Figure 5B). Traces of the H-zones were hardly visible. We need to point out that the histological changes started significantly later in the control samples – after 14 days, when the pH values decreased, the effect of the muscle tissue endogenous enzymes was manifested and the brine diffused deep into the meat pieces.

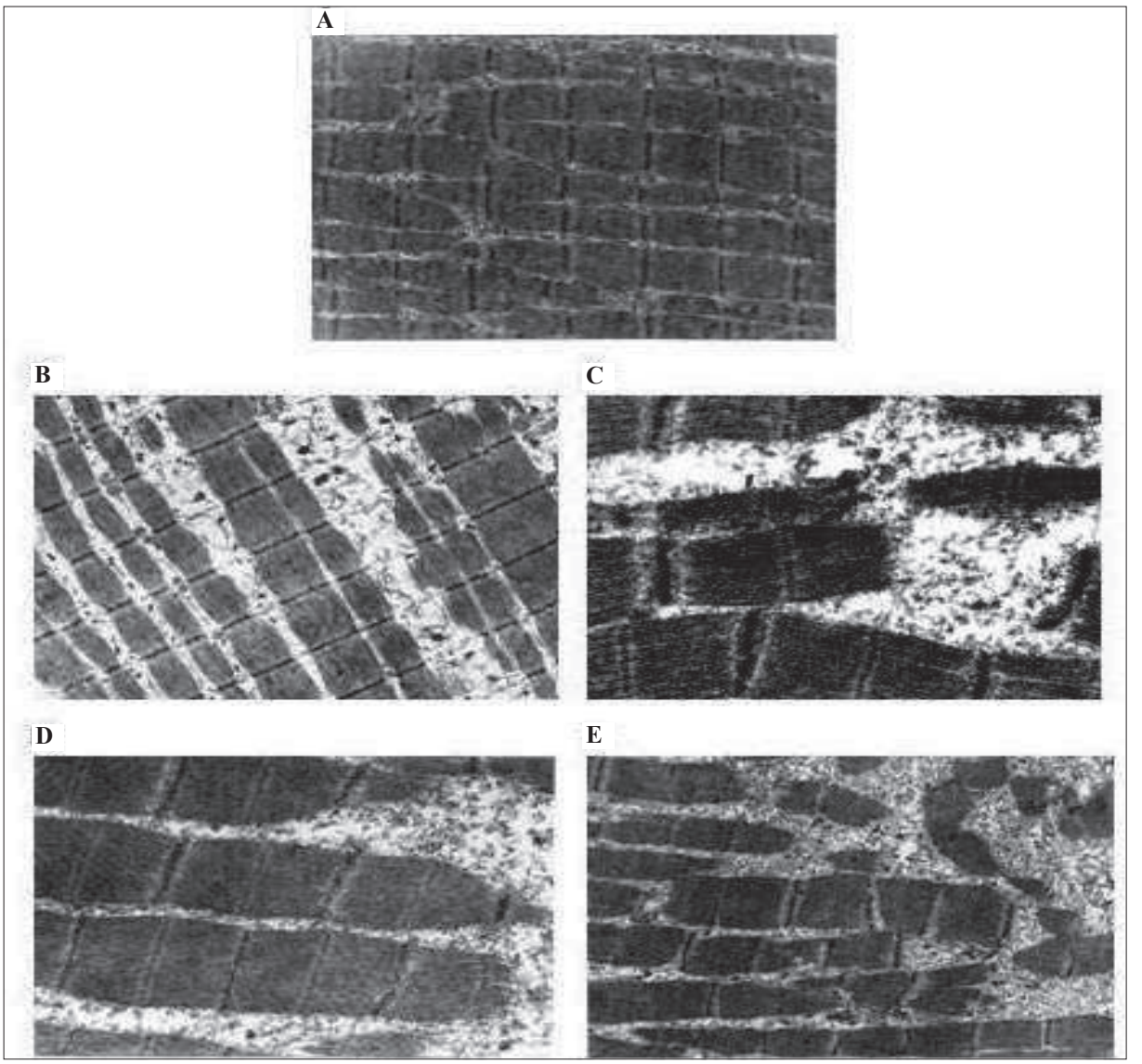

Fig. 5. Changes in the microstructure of the samples produced without (C) and with a starter culture (MT2): A – raw meat, 10 000 x magnification; B - sample C on 14 day of ripening and drying, 10 000 x magnification; C - sample MT2 on 14 day of ripening and drying, 18 000 x magnification; D - - sample C on 21 day (final product), 14 000 x magnification; E - sample MT2 on 21 day (final product), 10 000 x magnification

During the ripening and drying process, the microstructural changes in the inoculated (MT2) Elena fillet were more pronounced and were due to the complex effect of meat proteases, the growth of LAB, Micrococcaceae and the products of their activity (Figure 5 C, E). In these samples the microstructure started changing as early as the first days of technological processing, which coincided with the growth of the introduced LAB. The proteolytic activity of the endogenous enzymes complemented by that of the microbial culture caused destruction of the endomysial layers of the sarcolemma thereby enabling the penetration of the salting materials and the starter into the interior of the muscle pieces. There they hydrolised the microfibrillar and sarcoplasmic proteins and the affected muscle fibres acquired structural characteristics of different extent and size.

The ultrastructural changes in the test and control samples at the end of the drying and ripening process showed that a longer technological processing resulted in significantly greater changes in the myofibrils of the dry meat products. The meat proteins were hydrolysed largely, which was also confirmed by the results obtained by examination of the electrophoretic profiles of the samples (Figure 4). Deformation and rupture of the protofibrils were visible at several places in the control samples, which indicated the initial stage of proteolytic effect of tissue enzymes upon myofibrils (Figure 5B, D). It was interesting to note that the muscle fibres were unevenly affected by the enzyme activity and some fibres remained intact. Under the effect of the salting materials and enzyme systems, the muscle fibre myofibrils were hydrolysed to varying degrees. The H-zones and M-lines could be seen relatively well at a number of places. The histological changes occurring in the test samples may be viewed as the result of changes in two aspects: accelerated acidification under the effect of the bio-consortium starter culture which led to lower pH values activating the endogenous enzymes, and the proteolytic activity during the ripening of the strains used, i.e. *L. plantarum*, *L. lactis* and *M. varians*. Similar results were found by other authors (Casaburi et al., 2007; Fadda et al., 2010).

Sensory characteristics

The analysis of the sensory evaluation results (Figure 6) showed that no significant difference was observed between the test and control samples with regard to appearance. A statistically significant difference ($p < 0.05$) between the test and control samples was found with regard to colour, ripened flavour, taste and texture. The cut surface colour of the test samples was given a statistically discernible higher score compared to that of the control samples and confirmed the results of the objective measurements of the colour characteristics. The higher scores in the sensory evaluations of texture, taste and ripened flavour were consistent with the results on the changes in the protein fraction established by biochemical and microstructural analyses. The overall sensory evaluation of the samples provide the basis for a conclusion that the bio-consortium starter culture used improved significantly the quality characteristics of the final Elena fillet Bulgarian dry cured non-comminuted meat products compared to those manufactured according to the traditional technology.

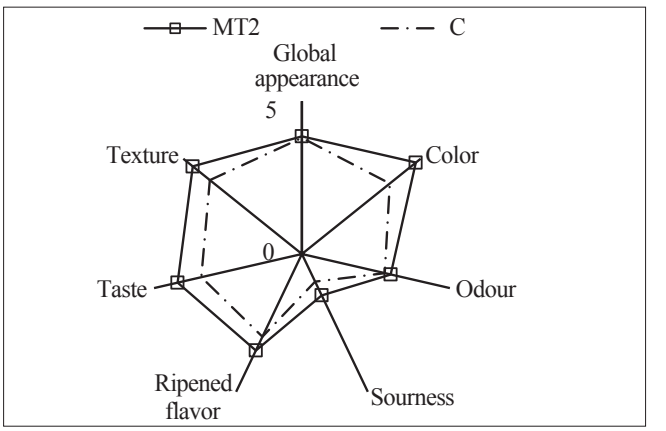

Fig. 6. Sensory evaluation of final dry cured Elena fillets produced without (C) and with a starter culture (MT2)

Conclusion

The *L. plantarum*, *L. lactis*, *M. varians* strains included in the composition of the bio-consortium starter culture showed good adaptability during the manufacture of Elena fillet Bulgarian dry cured meat products and effectively contributed to the faster and more significant lowering of pH values. The starter culture affected the biochemical changes by enhancing the colour formation processes and intensifying proteolysis in meat proteins during drying and ripening. The accelerated acidification activated the endogenous proteases of the muscle tissue and, together with the proteolytic activity of the bio-consortium starter culture, changed considerably the microstructural characteristics of dry cured meat products. This resulted in destructive changes in the actin and myosin protofibrils. These changes affected the formation of a better complex sensory evaluation of the inoculated Elena fillet samples compared to naturally fermented products.

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