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THE COMPLEX INVESTIGATION OF THE GOOSE LIVER DEVELOPMENT

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1. Background of research, Objectives

During the past years the economic environment of waterfowl breeding has shown a definite change. On the biggest buyer's market, in France, the production and acceptance of fatty duck liver is increasing continually, compared to goose liver.

Besides the species and hybrids specialized on meat production, the breeders raised such special liver producing hybrids that well tolerate large-scale production circumstances as well. Hungary has been the first among producers for years with its 1800 t / year production, 75 – 80 % of which is exported. Due to the successful breeding in case of the hybrids brought into production, the average yearly liver weight increase is 0.5 % with respect to the results of the past ten years.

The animal protection regulation of EU allows the trade of goose liver produced with force-feeding until 2011, after that only alternative solutions will be acceptable, being totally unknown for the Hungarian practice today. On the basis of all these those new methods must be found that are profitable from the producers' view, and that ensure proper quality goose liver production for the consumers.

In my dissertation I tried analyse goose liver in a complex way from several aspects. According to this I stated my research aims as follows:

- Adaptation of *in vivo* CT examination methods in order to follow-up the liver development, and evaluation of pixel density values based on PLS regression in order to estimate the fat content.

- Characterization of force-feeding liver production based on certain blood biochemical parameters and liver histological imaging.

- Development of a fast examination method based on NIR spectroscopy to qualify liver as a product.

2. Materials and Methods

Our examination was carried out on 240 (both sexes) Grey Landes geese. In case of the birds raised with intensive breeding technology the rearing phase preceding force-feeding was divided into two parts: 0 – 6 weeks of age and 7 – 14 weeks of age (pregavage).

After the rearing period the birds over 4.2 kg, independently from their sex, where placed out to a force – feeder. In the first 4 weeks of the rearing the birds were fed with commercial pelleted goose feed, then in the last two weeks with a rearing feed. The feeding preparation was restricted in time and in the so-called *ad libitum* phase the birds were given rearing feed.
An exception from this was one case when in the latter mentioned phase the rearing was changed with force-feeding diet. The birds were not restricted in their water intake.

During force-feeding the animals fed on simmered corn prepared by the feeder. The traditional force-feeding took 18 to 21 days in a way that on the first two days the birds were force – fed four times a day, then six times a day. In case of the decreased force-feeding number technology which took 18 days the birds were force – fed 3 times a day during the whole period of the treatment.

2.1. Experiments

2.1.1. CT examination of liver development with methodological aim

The pregavage technology lasted until the 15 weeks of age. Between the 18 and 20 weeks of age in the so called regeneration phase the birds ingested only water, but not feed in spite that they could eat *ad libitum*. In the first five timepoints CT cross - sectional digital images were acquired about all the 3 animals. At the age of 20th week of age only two birds were examined.

N	3
Times of examination	under rearing: 11th, 15th, during force - feeding: 16th, 17th, 18th,
	during regeneration: 20th last day of week
Samples	liver
Type of examination	tracing of liver tissue infiltration with CT scan, 3D reconsruction

2.1.2. CT based estimation of liver fat content

In the course of repeated CT scannings, the examined samples decreased with 8 animals each time because of their experimental slaughtering.

N	32
Times of examination	under rearing: 14th, during force - feeding: 15th, 17th
	during regeneration: 19 th week
Samples	liver
Type of examination	measurement of liver fat content chemicaly and its estimation with CT scans

2.1.3. Examination of the effect of force-feeding preparation

Ten of the examined animals were fed with commercial pelleted goose feed and 6 with force-feeding diet. According to this the feeding technologies were the same, but the composition of feed was different.

N	16
Times of examination	at the end of pregavage: 14th week
Samples	liver, blood serum
Type of examination	analysis of CT scans, definition of chemical compounds
	examinations serum metabolites and emzymes

2.1.4. Examination of force-feeding

In this examination we applied a complex approach and carried out measuring based on five different principles. The number of animals decreased each time with 6, due to the slaughtering.

N	30
Times of examination	under rearing: 6th week, during force - feeding: 7th, 11th, 14th, 18th day
Samples	liver, blood serum
Type of examination	CT scans, chemical composition, membrane fatty acids
	histological sections, serum metabolites and enzymes

2.1.5. Ultrasound test

Several kinds of examination heads were applied in order to determine the overlapping of the right liver, as compared to the rib. In the course of the US

test the birds were put on an examination stand in order to bring the liver into a proper position.

N	35
Times of examination	at the end of the force - feeding: 17th week
Samples	liver
Type of examination	liver size, liver weight measurement with US scanning

2.1.6. Examination of liver production with prolonged force-feeding period

In the first examination period we slaughtered 20, then each time 7 – 11 birds. The interval between 19 and 22 days can be considered as overfeeding, our aim with this was to produce a so-called "fatty liver" of questionable quality.

N	68
Times of examination	under rearing: 14th week, during force - feeding: 13rd, 18th day,
	during regeneration: 19th, 20th, 21st, 22nd day
Samples	liver, blood serum
Type of examination	analysis of CT scans, determination of chemical composition
	examinations on serum metabolites and emzymes

2.1.7. The application of the NIRS method in the evaluation of liver fatty acid composition

We purchased the liver from Merian Orosháza Rt. especially with the purpose of product examination. The livers were brought into the laboratory after factory qualification, with average weight betwen 692 ± 168 g. The force-feeding took 18 days the rearing and feeding conditions met the widely applied intensive technology in Hungary.

N	50
Times of examination	during force-feeding: 18th day
Samples	liver
Type of examination	NIR spectra, raw sample
	chemical composition, fatty acid profile determination

2.2. Applied examinations method

2.2.1. Ultrasound test

The two main lobes of the liver due to their different shape during expansion period can be seen at different times with ultrasound (US). It is the right lobe that grows first over the ribs the latter making the examination difficult. The lobes outlapping length was examined with ANISCAN type US equipment, trying 5 types of head. We conducted the US scanning under farm conditions and fixing the animals on examinations stands developed by us for this purpose, without applying anaesthetics.

2.2.2. CT scanning and image evaluation

We conducted *in vivo* CT scanning of the geese at the Institute of Diagnostic Imaging and Radiation Oncology on a Simens Somatom S40 spiral CT scanner. After weighing the birds one by one, we fixed the birds during examination on special containers with bands, with their legs pulled back and without applying anaesthetics. Due to the different size of the geese about each animal 8 – 30 slices, each 10 mm thick, were taken with total overlapping, covering the total liver region. In order to take 3D scans we applied 5 mm slice thickness and 5 mm steps. We analysed the scans with postprocessing program developed in above Institute.

2.2.3. NIRS examination

After homogenisation we acquisited near infrared spectra of raw livers with a FOSS NIRSystems 6500 (Foss NIRSystem INC., Silver Spring, MD, USA) equipment. During this reflection spectra were taken in the 1100 – 2500 nm wavelength interval with 2 nm pace, with the help of WINISI II v1.50 software. We used Sample Transport Module sample treating unit and a so-called Small Ring Cup sample holder with Full method for the examination.

2.2.4. The examination of the chemical composition of liver samples

The examination of the fat was conducted, according to MSZ 6830-6:1984, while the protein definition according to MSZ 6830-4:1981, and dry matter content was measured according to MSZ ISO 1442.

2.2.5. Definition of fatty acid composition

The preparation of the liver samples, then the extraction of the lipids was conducted in each case with the method of Folch at al. (1957), the determination of the fatty acids after being transformed into methyl esters and after gas – chromathographic separation was conducted with flame ionization detector (MSZ EN ISO 5508-1992).

2.2.6. Clinical chemical analysis

The different serum metabolite concentrations and enzyme activities were determined with a Konelab 20i automatic equipment with specified reagents at given wavelengths.

2.2.7. Histological evaluation

The segments embedded in 5 µ thick paraffin were stained with silver nitrate and haematoxilin – eosin method. With the haematoxilin – eosin method the saturation of liver cells with fat can be evaluated, while the silver nitrate staining method makes it possible to examine the membrane of the cells. The segments were characterized description analysis in the course of which emphasis was put on the degree and character (little or big drop shape) of fattening and the percent tactness of liver cells. The extent of inflammatory processes and the examination of the completnes of the cell membrane was evaluated as well, latter on the basis of the silver nitrate staining.

2.3. Data evaluation and statistical analysis

The correlation analysis between the results of the chemical analysis and the CT data were made with „Pearson" correlation analysis. In the course of the regression examinations we applied approaching based on linear and "S" diagram. After establishing variance analysis applied in the experiments the significance of the differences between the groups was examined with Tukey's post-hoc test. SPSS 10. software was used for the statistical analysis.

2.3.1. Evaluation of pixel density values

Equations to estimate liver composition were established with PLS regression based on X – ray density values. The construction of 3D histograms examining tissue distribution was made by the negative exponential interpolation method. The construction of 3D histograms was made with SYSTAT 5.01 software NEXPO algorithm as well as TableCurve (1998) 3D software, and the surface was made by the NURBS smoothing method.

2.3.2. Analysis of NIRS spectrum

Spectrum analysis was carried out with WINISI II version (2001) spectrum evaluating software. The relationship between spectra and reference qualities was analysed with the modified PLS method. The calibration was based on the second derivative of the total spectra. Gap and smooth values of four were applied with the application standard MSC. For the calibration testing leave–one–out cross validation was applied. In the course of mPLS of the model optimalization in case of each constituent component that factor was taken into consideration where the validation standard error was the lowest.

3. Results

3.1. CT examination of liver development with methodological aim

As a result of the methodological *in vivo* spiral CT test, the change of the pixel density values, that of the liver volume and surface, as well as average X – ray density values were described in the course of force-feeding, and latter, during the regeneration phase. Due to the applied scanning technics the morphology of the liver geometric condition was shown with 3D reconstruction. According to the results, the volume and the surface values measured at the 11th week of age at the begining of the force-feeding were highly similar. After the early fast increase by the 21st day of force-feeding that is by its end the liver volume quantyled (150 – 800 cm^3). Two weeks after the end of force-feeding during which birds did not intake fed only water inspite of ad libitum feeding, the liver volume reached the starting value as well. The measured density values (HU) followed the changes

during force-feeding considerably and reversibly (from +80 HU it decreased to -55 HU then by the end of regeneration it reached +55 HU).

3.2. CT based estimation of liver fat content

The liver fat content was estimated with different methods based on the pixel density values. Fist correlation was calculated between the average and the most frequent HU values then between the calculated HU index (so-called fat index) and the chemically determined fat content. From the point of view of the fat estimation among the CT scan parameters the closest relation was given by the most frequent HU values. Further on multivariate linear regression equations were established to estimate the fat content determined with chemical analysis as independent variables with the involvement of the measured and calculated CT parameters.

3.3. Examination of the effect of force-feeding preparation

In the course of model testing so-called pregavage method was applied. Since the technology and the applied species were the same, the effect of feeding diet and rearing pelleted diet on the liver could be compared. According to my results besides the average HU values a significant difference was obtained relating to all the measured data (liverweight, livervolume, DM, fat and protein content belated to 100 g DM). After blood sampling statistically significant difference was found in case of albumin, TG, total bilirubin, uric acid as well as the main enzymes LDH, GGT and amilase. They refer to an advanced intensity metabolism of both the measured metabolites and enzymes showing the effectiveness of preparation with force-feeding diet. At the same time, it does not mean the applicability of the technology to produce liver without force-feeding, but calls attention to that this method was able to influence the size and the fat content of the liver.

3.4. Examination of force-feeding

In a new way the tissues taken at the testing times were classified with histhologic method on the basis of the micro- and macrovesicular fattening of the cytoplasm. Parallel with this, in case of characterization of the

metabolic status of force-fed geese with serum parameters we found the increase of serum TG, HDL and total cholesterol.

The AST and ALT activities also increased at the same time, neither of these intracellular enzymes showed liver functioning disorder or a change to the cell membrane. Joint evaluation of the histological image and serum parameters was also conducted taking into consideration the phospholipid quantitative feathures of the cell membrane. It is intreasting that a significant cell damage was not found in the last phase of force-feeding which proves the good technological tolerance of the Grey Landes goose.

The force-feeding period took 18 day to test in the course of which the liver fat content increased continualy, finaly it exceeded 50 %. The protein content of the liver was just the opposite. In respect of fat content the process was made numerical with 3D histograms constructed on the basis of the density values. In case of the liver fat saturation certain tissue images first the samples at disposal from the 18th day of force-feeding were classified on the basis of different level fattening. Interestingly the degree of the fattening effected its stereophonic organisation as well. Characteristicaly the steatosis evolved according to the following steps micro and macro vesicular lipid state, finaly cysta fattening resulting from the liver of the macrovesicular structure could be practised. At the same time based on the segment evaluation cell rupture which is important from technological point of view could not be justified.

During testing the serum parameters are aim was to characterisation to metabolic status of the birds during force-feeding analysing it joithly with the previously described tissue image. While the concentration of the total protein (55.8 – 64.7 [g/L]) and of the albumin (21.0 - 19.5 [g/L]) did not change significantly during treatment, the uric acid concentration of the serum (in close connection with the protein content of the feeding) increased significantly by the end of force-feeding. Because of the high carbohydrate was observed during testing. The activity value were betwen 2400 and 3000 IU / L accordance with the normal physiological rate.

After this the histological evaluated samples were devided into two categories accordig to the fattening value of the segment. In case of the two groups the wight and the fat content of the livers were definitely different. When analyzing the serum parameters we found that the activity of serum

ALT is in close connection with the above categories. It is probable that beside the intensive fattening process some cell damage must also be taken into consideration, which although can be measured but from phisiological point of view it cannot be considered critical.

The phospholipid quality features of the cell membrane were also tested (fatty acid profile) with regard to two groups. Based on the advanced fattening practice on the tissues histological images it can be assumed that the cell TG accumulation may probably lead to cell damage. Interestingly in the fatty acid profile of the phospholipid fraction of the liver tissue difference was found in case of only one fatty acid (C 18:0) among the fattening categories based on histology. According to our result it can be assumed that in case cell damage must be taken into consideration it may take place not on the lipid level but on the membrane protein level in case of force-feeding. As a summary it can be stated based on the quality analysis of the liver cell phospholipid as well as based on the blood samples taken at this time an significant cell damage does not occur in the liver kept its structure well an was of excellent quality from technological point of view.

3.5. Ultrasound test

Ultrasound test examination was also conducted besides CT scanning in order to evaluate a fast examination method that can be applied among factory circumstances, applying the phenomenon that during the fattening process of the geese the right side lobe laps over the rib fast than the heart – shaped left one. This caudal overlapping makes it possible far the US test to sense it and to measure its length. During pre-measuring the testing technology was evaluated including the selection of the US examination head and we applied frequency as well as a stand was also developed for the proper positioning. Based on the data of the cut following the in vivo testing in order to test the US methodics the size of overlapping and the liver weight were estimated (R^2 = 0.9; 0.86). Based on the above results the method is considered suitable to categorize the stock at the end of the pregavage.

3.6. Examination of liver production with prolonged force-feeding period

After analysing the change of the serum parameters it was found that the total cholesterol, is like an indicator of the other serum features in case its

increase is compared with higher total protein, TG, uric acid, LDH, GGT values. It can be stated on the level of the birds that those animals with higher metabolic and enzymatic value did not show liver weight value different from the average at the same time could be characterized with below average fat content.

It is probable that the geese with extremely high serum lipid content may not react properly to force-feeding. Parallel with this the lipid storage ability of the liver is lower and in this case the blood lipid circulation increases. It is interesting that significant difference could be found neither in the liver weight nor in the chemical compounds, nor in the serum parameters during overfeeding, when the 19th and 22nd days of the force-feeding were compared. On the bases of this it can be assumed that more days are necessary to reach this state.

Their liver fat content did not increase after th 18th day (over 350 – 400 g liver weight) a kind of saturation process could be observed. When examining a relation between the liver weight and the chemically determined fat content the best approach ($R^2 = 0.91$) could be reached in case of the application „S" curve. Based on the pixel density frequencies determined during in vivo tests with the help of PLS regression the R^2 value of the calibration equations relating to the liver protein and fat content was over 0.97 in case of both components.

Besides describing the metabolic status relation were searched between the liver tissue features estimated in vivo and the blood serum parameters. The correlation between the blood lipids and estimated the liver fat content with CT was r = 0.68; 0.79; 0.82 in the order of the TG, the total and the HDL cholesterol. The cholesterol concentration of the serum was the relatively high with respect to the birds' force-feeding as well as high fat intake. When the birds were grouped according to test-days based according to the above (18th, 19th, 20th, 21st, 22nd force-feeding days) the higher total cholesterol values were companied with higher total protein, TG, uric acid an total bilirubin concentration over the average in each case. Similarly to this the LDH and GGT activities were far higher than the group average. A strong correlation relationship was found between the total and HDL cholesterol concentrations (r = 0.9).

The examination of the enzymatic adaptation had interesting results. From among the intracellular enzymes in the serum the highly increased activity of the ALT and AST can refer to liver functioning disorder or may be cell membrane damage. The activity values did not show other changes until the 22nd day of force-feeding. In case of both enzymes our experience was that in the group of the force fed birds besides the significant increase of the average value the dispersion also increased significantly. During the experiment the ALT activity doubled while the AST activity quadrupled. A slightly increase was seen in case of LDH while the GGT change was not systematic during force-feeding. According to my result some birds showed bigger hepatic sensibility or smaller tolerance in this respect. It is probable that in this case the GGT which is applied as an indicator of hepatopathia in human diagnostics was not an informative indicator in our case, at the same time it is probable that the activity of enzyme increases significantly in the last phase of force-feeding which can be characterized with intensive fat storage.

3.7. The application of the NIRS method in the evaluation of liver fatty acid content

With respect to fatty goose liver as a product, samples categorized during factory qualification were examined with NIRS and gascromatography method. The multiple saturated and unsaturated fatty acid ration in the force – feed liver is far over what is recommended in case of animal origin food stuffs. It was true about n6 / n3 ratio as well, which was around 10 in the liver compared to the 3-4 value recommended from human health protection view. The judgment of the unsaturation index is double-sided from the point of view of healthy nutrition the measured value is quite low but from the point of view of storage it is beneficial because it refers to low sensitivity to oxidation. Besides the estimation of the fat content $R^2 = 0.81$ the fast examination method based on raw homogenised livers proof to be successful in case of the two fatty acids – oliec acid and palmitic acid – which are present in the biggest rate. The stearic acid, wich occurs in 15.5 % could not be estimated. Similarly well could be estimated the UI value as well which proofs the relatively high oxidative stability of the liver lipids.

4. Conclusion

The criticism of animal protectors in connection with fatty goose liver production resulted in the change of legal ruling as well as in the coming into force of the EU's prohibitive declaration from 2001. From this point of view those technological developments are considered important by me, which concentrate on liver production without force-feeding. In the course of my own examination I presented new facts in connection with the volumetric increase and fattening of the liver and I looked for those relations that might help the evaluation of new alternative technologies.

At the same time I wish to highlight the benefits of the non-invasive measuring methods (CT, UH) applied by me in case they harmonize with the more and more strict animal protections regulations. According to this kind of examinations can easily be authorized and repeated, and the results obtained in the course of them can be published.

The base of the market judgment of fatty goose liver is its size and fat content. The chemical components of the whole body of different farm animals were determined in the course of earlier, CT examinations in Kaposvár. In my own examinations, adapting the earlier results I applied different methods to estimate the fat and protein content of the liver. Fatty goose liver as an extreme high fat content product could to be very well characterised on the basis of variables deriving from the density frequency of pixel densities. Since at a given time the liver tissue can be considered almost homogenous, its compound can be estimated based on a few scans. Similarly to the examinations carried out on other animal breeds, we used up the facility of in vivo CT examinations in case of goose breed, which is based on the repeated scanning of the same animal. The method ensures the tracing of the building in of the liver in the course of the whole force-feeding. The non invasive CT digital imaging techniques can be a base for a selection method which is aimed at the increase of the liver output which in the near future - because of a strict ruling aimed at fatty liver production - can play an important part in the selection of those species that are suitable for liver production without force-feeding. The so called pregavage method is and has been developed because of market considerations so that high fat content liver with "foie gras" quality can be produced for those countries in which liver trade produced with force-feeding is banned. During the temporary period until 2011, with the use of the previously mentioned technology, the

geese can be examined at the end of the preparation phase. The birds with proper size liver which can be sold on the given market will be cut, while the other birds can be force – fed or after 2011 they can be sold as a commerce product.

The 3D reconstructions based on sequvenation cross sectional digital imaging well characterize the morphology of the liver. The available images make it possible to set up a kind of geometric model which would make it possible to compare the liver infiltration of different goose genotypes during force-feeding.

I think that the application of UH in liver production can be very important, especially in the examination of liver force – fed in an alternative way. According to my results this method can be considered a kind of pre-selection method, so it can take part in the selection of those animals that have a good reaction of pregavage. In the course of the outlined factory experiment we tried and proved the incorporation of the method into technology.

The complex examination of force-feeding called attention to that the modelling of those processes which are connected to the formation of overfed liver takes a longer force-feeding period. Although in our examinations the fat content of the livers was near to the critical 55 %, neither the serum parameters, nor the analysis of the tissue samples showed the damage of the liver cells. Based on this, taking into consideration the tolerance of the used grey Landes genotypes, further examinations based on longer force-feeding period are recommended. It must be stated here that due to the previously mentioned changes in ruling, at least in Europe, the practical usability of the expected result is low.

Since alteration in the membranes was not found with gas chromatography analysis of the fatty acid composition of the phospholipid fraction, more examinations focusing on membrane proteins are necessary.

From the point of view of the processing industry, knowledge of the chemical compound and especially of the fat content of the liver samples of the animals that are slaughtered at slaughterhouse is vital.

According by it is important to create a fast examination method which used in big slaughter houses, can be made suitable for on – line operation. For this

purpose the NIR methodic is considered suitable because it works solvent free, so it does not pollute the environment, and compared to the orthodox methods it can be used to carry out routine examinations with big sample number at much lower costs. As a result of our examination as well as on the basis of the features of the portable NIR equipments that appeared on the market, every facility is available to use the method.

During our experiments the NIR method proved to be suitable to measure the unsaturated index in the liver and through this to judge the oxidative stability and finally the perishability, which can be important from food technological view.

My result can be applied in the production of the European fatty poultry liver, 95 % of which is based on the use of mulard duck. At the same time watching the international trend of liver production I do hope that my examination results might be of interest for Far – East researchers as well.

5. New scientific results

- I proved with the use of spiral CT cross sectional digital imaging method the reversibility of the liver volume and fat content change in the regeneration phase, following the force-feeding.

- I worked out a method based on PLS regression with the use of the X-ray density distribution values of pixels to estimate the fat content of the liver *in vivo*.

- I stated that in the course of force-feeding the excessively increased total cholesterol level of the serum is accompanied with the advanced activity of the serum's LDH, GGT, and with the increased concentration TG, total protein, uric acid in case of those animals that have smaller liver weight compared to the population average.

- I stated that the fatty acid profile of the fractioned, mainly membrane origin phospholipids content of the total fat content slightly and strongly fatty livers is only slightly different, referring to the preservation of the cell integrity

- I elaborated a NIRS based fast examination method to estimate the chemical composition of fatty goose liver

6. Scientific papers and lectures on the subject of the dissertation

6.1. Articles in foreign languages

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