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APPLICATIONS OF THE HISTOMETRIC METHOD ASSISTED BY THE COMPUTER IN THE AVIAN DIGESTIVE TUBE INVESTIGATION

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SUMMARY

The objective of this research consisted in the use of a histometric method by analysis of computerized image on the epithelium of the small intestine mucosa in broiler chickens for the testing of the prebiotical effect of mannanoligosaccharides (Bio-Mos, Alltech). There were made assessments about the significance of the differences between the avian groups for four parameters (height of villi, the width of villi, the number of goblets and the depth of the crypts) in fragments sampled from two areas of the ileum. The obtained values were statistical processed. The results comparison of the two types of samples (A and B) revealed significant differences on the number of goblets ($p < 0,001$), the height of the villi ($p < 0,01$) and the width of the villi ($p < 0,05$). The analysis of the obtained results between the animal groups was not conclusive because the comparison group was not known. The research conducted established the morphometric diagnostic method on the intestinal mucosa in birds, being the first paper on histometry application in veterinary medicine in Romania..

Morphometry (MM) uses methods derived from histology for the identification of the effects of some toxins, drugs, stimulant preparations and the establishment of some values of the cellular parameters in malignant and benign tumors. The MM method analyzes from the quantitative point of view different tissues and cells.

Traditional, the histologists rely on a verbal description of the tissue structure, sometimes approaching a mathematical description.

In recent years, at the international level, the measurement of the tissues microscopic images, known as histometry (HM) was used increasingly both in research and diagnostic. The histometry methods represent a wide extension of those borrowed from geology and metallurgy.

An important part of the HM technique is that it allows 3D measurements which derived from the 2D microscopic images provided

by histological sections. Some of the methods are based on complex mathematical calculations.

The development of the computer equipments, entered in the management systems of the cytopathology and histopathology laboratories, became progressively since 1990 increasingly common in industrialized countries (Morenes et al., 1992; Anderson şi Lowe, 1992; Barteles et al., 1992)

HM by computerized image is a height tech of histology, representing a relatively young field for the veterinary medicine, its impact gradually evolving in the cancer, immunopathologic and endocrine pathology, given by the height level of specificity that it offers. For example HM is more sensitive and specific than the HE or Giemsa stain for the histologic analysis of the subtypes of follicular lymphoma or for the establishment of villi height and width, the number of goblets and the depth of the crypts from the small intestine (Preston et al., 1990; Radecki et al., 1992; Bradley et al., 1994)

Internationally the HM technics are currently possible in the lab diagnosis of tumors in humans. In animals this method is used in the diagnosis of tumors and the research for the action establishment of the pre- and probiotic and immunomodulatory preparations.

The gastrointestinal tract (GI) is permanently invaded by foreign substances some of them harmful and the *lamina propria mucosae* is a place for proliferation both beneficial and disadvantageous of the bacterial flora (Mitjans et al., 1998; Adeola şi King, 2006)

The correct measurement of the absorption rate in the microvilli (brush edges) on the surface of the enterocytes and the measurement of the intestinal villi for different nutrients in mammals, birds and reptiles is important and effective in numerous research projects in terms of the digestive, ecological, nutritional function and the functional plasticity of the bowel (Starck et al., 2000; Karasov şi Diamond, 1983; Garcia et al., 2006; Dibner et al., 1996; Strong et al., 2005).

This work presents aspects of morphometry by analysis of computerized image on the epithelium of the small intestine in broiler chickens treated with mannanoligosaccharides (Bio-Mos, Alltech) for the testing of the prebiotic qualities of the product.

There were determined the number of goblets, the height and the width of the villi and also the depth of the crypts.

The researchers of the early 90 have found a component of yeast cell wall – the mannanoligosaccharidic protein – phosphorylated which is an effective alternative to antibiotic growth promoters. The oligosaccharides from yeast in which mannose is the primary

carbohydrate (MOS), proved active in the digestive tract in several animal species (Spring şi Pîrvulescu, 1998; Considine şi Spring, 2000; Spring et al., 2000; Spring, 2002, Kocher, 2006). This prevents pathogens to colonize the digestive tract and prepare and modulate the immune system of animals giving the opportunity to respond quickly to infection (Spring şi Pîrvulescu, 1998; Considine şi Spring, 2000; Adeola şi King, 2006).

The research has been aimed at the development of modern diagnostic methods and testing by histological and morphometric techniques by the analysis of computerized image applied to the epithelium and lymphoid structures associated to the mucosa (*mucosal associated lymphoid tissue* –MALT) in birds.

1. MATERIAL AND METHOD

Intestinal morphometric examinations were conducted at 40 broiler chicken, in the age of 37 days treated with an extract prepared from the outer cell wall of *Saccharomyces cerevisiae* (Bio-Mos, Alltech). 40 fragments were taken with *Meckel diverticulum* (Md) A and 40 fragments with the cranial portion of ileum 30 mm from the ileo-caecal junction (B).

The experiments were carried out at the Animal Nutrition Institute in Zurich, Switzerland, under a contract research on four lots of birds (the lots I, II, III, IV) and in each group were allocated 10 animals each. After slaughter were taken by two pieces of small intestine from all broilers and all experimental lots. The fragments were fixed in 10% neutral buffered formaldehyde solution and sent to the Pasteur Institute in Bucharest, Romania for histometric processing.

They have not submitted information regarding the treatment schedule of broilers treated with the Bio-Mos preparation and was not known how were choosen the lots.

The histological technique and the investigated parameters were similar to those used (by classical method) in works by Bradley et al., 1994 with some modifications which we present in the lines below.

- The pieces inclusion has been made in Paraplast plus (Sigma);
- The staining of the sections has made by the periodic acid-Schiff (PAS)- alcian blue (AB) reaction, pH 2,5 (Serva).
- The measurements were performed under a computer-assisted microscope.

Following the histological fixation (10% neutral buffered formaldehyde) the pieces were treated with alcohol and dehydrated in

toluene in the Citadel device 1000 – Shandom (2000), and the inclusion has been made in Histocentre 2 device – Shandon (2000).

The histological preparations were examined under the light microscope Nikon (Labophot type) fitted with a Sony color video camera (CCD-IRIS/RGB) which processed images and transmitted them to a computer equipped with software Lucia M 3.00b/2001 image processing.

This program allows measurement with an accuracy of up to 1/1000 μm of the size of various cells by transforming pixels in μm.

Before the start of the measurements the objects standardisation was performed. With the aid of a micrometer object it has been determined the number of pixels corresponding to a micrometer for the 4, 6, 10, 20, 40 objects, and the computer was programmed to read directly in the micrometer size. After the mathematical calculations, the values obtained were converted from μm in mm.

The establishment of the number of goblets, of the height and width of the villi and also the depth of the crypts were determined on 9 villi from each sample as shown in the Bradley şi col., 1994) method, to mention that reading the samples was performed with a computer-assisted microscope. For the 80 samples of small intestine there were performed 2880 measurements.

The values obtained were statistically processed in the sense that were calculated for each parameter arithmetic average ($\overline{X}$), its standard error

(E.S. $\overline{X}$) and the coefficient of variability (CV% /lot) Wardlaw, 1993.

There were made assessments about the significance of the differences between the avian groups for each parameter by the "t" test (student). It was also calculated the correlation coefficient (r) between the height of villi and the number of goblets/mm.

The research conducted established the morphometric diagnostic method on the intestinal mucosa in birds, being the first paper on HM application in veterinary medicine in Romania.

2. RESULTS AND DISCUSSIONS

The data obtained were synthesized as both individual and group mean values for the samples collected from the M. d. area (A) and for those collected from the ileo-caecal junction (B).

I. The synthesis of the results provided by the measurements performed on broiler A batch of samples are presented in Table 1 and Chart 1.

Table 1

Values obtained in batches of A samples

Groups		Goblets (no./mm)	Villi height (mm)	Villi width (mm)	Crypta depth (mm)
I	$\overline{\overline{X}}$	52 ± 10.2	1.015 ± .159	.079 ± .009	.097 ± .024
	SE$\overline{\overline{X}}$	3.2	.050	.003	.007
	VC%	19	15	11	24
II	$\overline{\overline{X}}$	55 ± 7.25	.968 ± .173	.075 ± .009	.093 ± .027
	SE $\overline{\overline{X}}$	2.29	.054	.003	.008
	VC%	13	.17	11	29
III	$\overline{\overline{X}}$	50 ± 7.6	1.070 ± .156	.073 ± .009	.097 ± .019
	SE $\overline{\overline{X}}$	2.4	.049	.003	.006
	VC%	15	14	12	20
IV	$\overline{\overline{X}}$	48 ± 10	1.055 ± .120	.087 ± .015	.098 ± .021
	SE $\overline{\overline{X}}$	3.1	.038	.005	.007
	VC%	20	11	5	21

$\overline{\overline{X}}$ = Mean of means ($\overline{X}$) VC% = Variability coefficient/group

SE $\overline{\overline{X}}$ = Standard error of $\overline{\overline{X}}$

Because it was not known a comparison group (control) the significance of the differences was done for each batch and parameter over the other three groups.

From Table 1 it results that in group 1 significant differences have not been found in comparison with the other three groups (groups II, III and IV). In lots II and III the significant differences was only towards the group IV in the villi width parameter.

These findings are given in Chart 1.

Chart 1

Significance of differences among mean values (samples A)

Lot	I	II	III	IV
I		ND	ND	ND
II	ND		ND	ND /3 parameters; SD_{vw}*/1 parameter
III	ND	ND		ND /3 parameters; SD_{vw} **/1 parameter
IV	ND	*	**	

ND = Nonsignificant difference

SD_{vw} = Standard deviation of villi width

*SD_{vw}; $p < 0.05$

$t = 2.18$ for $LD = 18$

** SD_{vw}; $p < 0.05$

$t = 2.5$ for $LD = 18$

LD = Liberty degrees

From the chart above results that the variability was the middle order for three parameters (villi height = 11-17%, villi width = 5-12 %, goblet cells number = 13-20%); as for the cryptal depth, its variability was high (20-29%).

We present some photo shoots obtained from the small intestine samples prepared for histometric exams (photo 1,2,3)

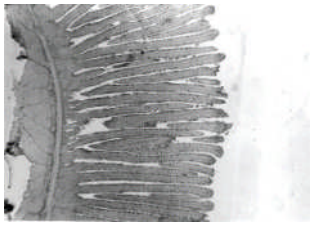

Photo1. Ileum (M.D. area). Numerous villi, intestinal crypts with submucosal glands, muscular and serous tunics. PAS – alcian reaction

Photo2. Ileum (M.D. area). Intestinal villi with numerous goblet cells. PAS – alcian reaction (x250)

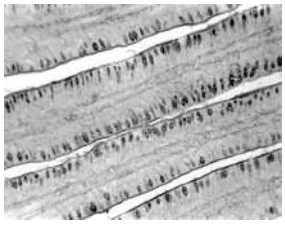

Photo3. Ileum (ileo-caecal junction). Intestinal villi with numerous goblet cells PAS – alcian reaction (x250)

As regards the correlation between the villi height and the goblets number/mm the following were found:

- Groups I and II it is distinguish a weak positive correlation (r = 0.276
and r = 0.439 respectively)

- Groups III and IV present a weak negative correlation (r = - 0.240 and
r = 0.383 respectively).

The values recorded for samples A show that in the case of Groups III and IV the total cell number remains unchanged, while the villi height is higher.

The evaluation may be that, with the Groups I and II, the total number of goblet cells rises together with the villi height increase.

II. The Summary results of measurements obtained from the portion of small intestine from the ileo-caecal junction (probele B) are presented in the Table 2 and Chart 2.

Table 2

Values per groups in samples B

Groups		Goblets (no./mm)	Villi height (mm)	Villi width (mm)	Crypta depth (mm)
I	$\overline{X}$	73 ± 13.3	.640 ± .062	.100 ± .009	.095 ± .021

	SE $\overline{\overline{X}}$	4.2	.019	.003	.006
	VC%	18	9	9	22
II	$\overline{\overline{X}}$	72 ± 15.9	.690 ± .115	.097 ± .012	.108 ± .027
	SE $\overline{\overline{X}}$	5.0	.036	.004	.008
	VC%	21	16	12	24
III	$\overline{\overline{X}}$	67 ± 11.6	.638 ± .111	.094 ± .010	.095 ± .010
	SE $\overline{\overline{X}}$	3.6	.035	.003	.003
	VC%	17	17	10	10
IV	$\overline{\overline{X}}$	70 ± 1.1	.648 ± .137	.102 ± .013	.102 ± .021
	SE $\overline{\overline{X}}$	3.5	.043	.004	.006
	VC%	15	21	13	20

$\overline{\overline{X}}$ = Mean of means ($\overline{X}$) VC% = Variability coefficient/sample

SE $\overline{\overline{X}}$ = Standard error of $\overline{\overline{X}}$

No significant difference was noticed, with samples B, on comparing each group with the other three groups.

A weak negative linear correlation was found between the villi height and goblet cells number in all the groups: Group I r = -0.375, Group II r = -0.569, Group III r = -0.592, Group IV r = -0,342.

We present some histological photo shoots obtained from the small intestine (samples B) prepared for morphometric technic (photo 4 and 5).

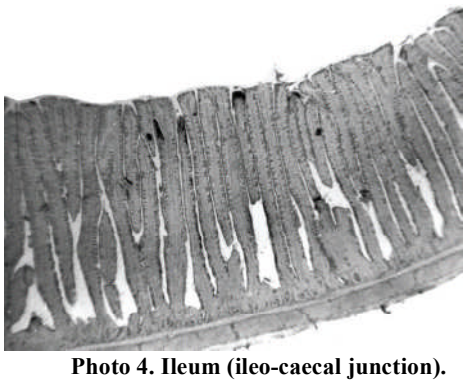

Photo 4. Ileum (ileo-caecal junction). Numerous villi, intestinal crypts with submucosal glands, muscular and serous tunics. PAS – alcian reaction (x140)

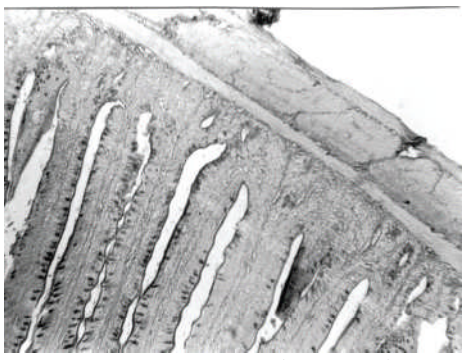

Photo 5. Ileum (M.D. area) Intestinal crypts with submucosal glands. PAS – alcian reaction (x250)

III. Samples A/B comparison

The significance of the differences among the values of the test parameters of groups A and B is presented in Chart 2.

Chart 2

The significance of the difference among means

Groups	Goblets (no./mm)	Villi height (mm)	Villi width (mm)	Crypta depth (mm)
I/IV	ND	ND	ND	ND
II/IV	ND	ND	ND	ND
III/IV	ND	ND	ND	ND
A/B	VSDt = 4.73 p< 0.001	VSDt = 7.05 p< 0.001	SDt= 2.35 p< 0.05	ND

ND = Nonsignificant difference
VSD = Very significant difference
SD = Significant difference

On comparatively analysis the results obtained the villi height was found to be lower with samples B (Table 2) than with samples A (Table 1).

With Group I, there were very significant differences ($p < 0.001$) for the following parameters: number of goblets, villi height and width.

In the Group II, distinctly significant differences ($p < 0.01$) were recorded with the goblet cell number, and very significant ones ($p < 0.001$) for the height and width of the villi.

Group III presented very significant differences ($p < 0.001$) for the goblets number, villi height and width.

In the Group IV very significant differences ($p < 0.001$) were found as far as the goblets number and villi height are concerned. Also, significant differences ($p < 0.05$) were revealed in way of the villi width.

The safety of the methods for interpreting the results of intestinal epithelium measurements is based on the integrity of the mucosal epithelium and enterocytes and on the execution quality of the histological preparations.

The ileum histological examination revealed in all the birds examined a normal aspect of villi with many goblet cells characterized by the presence of large vesicles. The epithelium of the intestinal mucosa is delicate, with long intestinal villi. In the submucosa were found many glands with normal glandular epithelium cells (photo 5). The simple prismatic epithelium covers the intestinal villi and goes down in the Lieberkühn crypts in whose lumen is lining. It has been noted many goblet cells PAS-positive or alcian positive irregularly

disseminated on the flanks of villi alternating with numerous intact epithelial absorption cells (photo 2).

In the histometrical examination no significant changes were found in the studied parameters (the villi height and width, the number of goblet cells and the crypts depth) in the sections from the birds that came from the A samples. In the B samples were recorded lower values of the villi height parameter compared with the A samples.

However, the comparative analysis of the obtained results on the analyzed parameters in A/B samples have highlighted significant differences ($p<0.001$; $p<0.01$; $p<0.05$), on the number of goblet cells, the villi height and width.

Numerous studies showed that the administration of Bio-Mos in the diet of broilers and piglets resulted in an increase in villi length and a reduction in crypts depth. The overall size of the surface absorption in the intestine and the reducing of the renewal rate of the epithelial cells in the crypts improve the nutrient availability for absorbing (Adeola şi King, 2006; Kocher,2006)

In the work that we made, we were not aware of all the elements on the experimental protocol in broilers and we could not conclude on the prebiotic action of the Bio-Mos preparation.

It results that the measurements performed on the ileum showed a significant difference in the A samples over the B samples in three parameters: goblet cells, the villi height and width

CONCLUSIONS

It were investigated by morphometric examinations fragments of small intestine from the treated broilers with the Bio-Mos preparation, Alltech.

1. The histometric test results have shown statistically significant differences of the parameters: villi height, villi width, number of goblet cells and the crypts depth on the fragments collected from the same subject with M.D. (A) compared with the obtained fragments from the cranial portion of ileum (B).

2. The registered values in the A samples demonstrates that the number of goblet cells remains the same (the Group III and IV) or increases (the Groups I and II) while the villi height is bigger.

3. In the B samples were not found significant differences between the groups, and between the villi height and the number of goblet cells it has shown a weak linear negative increase in all the groups (the Groups I, II, III, IV).

4. The comparison of the A samples results with the B samples results has shown significant differences about the number of goblet cells ($p<0.001$), the villi height ($p<0.01$) and the villi width ($p<0.05$).

5. The analysis of the obtained results in the groups of animals studied was not conclusive on the prebiotic action of the used preparation.

6. The studies have aimed the development of a diagnostic and testing method by histometric technic by analysis of computerized image on the epithelium of the small intestine mucosa.

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