

## Utilization of the Chicken Embryo Model for Assessment of Biological Activity of Different Oligosaccharides\*

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The effect of different oligosaccharides –  $\alpha$ -galactoside preparations from *Lupinus albus* seeds differing in sucrose content, raffinose and fructooligosaccharides on the growth of chicken intestine microflora and the hatchability and weight of the treated embryos were studied. The assessment of biological activity of these oligosaccharides was done *in ovo* on the chicken embryo model. The eggs of experimental groups containing twelve days old embryos were injected into the air cell with 0.2 ml of Ringer water solution containing 0.1763; 0.8815 and 1.763 mg/egg of an oligosaccharide preparation, while the control group was injected with 0.2 ml of Ringer water solution only. All oligosaccharide preparations in higher doses had an influence on chicken hatchability and increased bifidobacteria in the colon of two day old chicken. The number of bifidobacteria depends significantly on the kind of oligosaccharide preparation used and its dose. For all experimental groups, the number of bifidobacteria was significantly higher in comparison to the control.

Key words:  $\alpha$ -Galactosides, RFOs, raffinose, fructooligosaccharides, bifidobacteria, chicken embryo.

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The bacterial flora of the alimentary tract is important for correct nutrition and good health. It is composed of harmful, toxic and beneficial microorganisms. The bacterial profile is formed by the consumption of food containing proper levels of prebiotics which induce the development of beneficial microflora. Oligosaccharides are prebiotics and the subject of broad studies (TOMOMATSU 1994; GIBSON 1998; GULEWICZ *et al.* 2002). Oligosaccharides are widespread compounds in the plant kingdom. Up to now, the fructooligosaccharides (FOs) are the best known and most commonly applied oligosaccharides (HIDAKA *et al.* 1986; SPIGEL *et al.* 1994; JONG WON 1996; VAN LOO *et al.* 1999). In this respect, the lesser known compounds are  $\alpha$ -galactosides, also called raffinose family oligosaccharides (RFOs).

These compounds are  $\alpha$ - (1–6) galactosides linked to carbon C-6 of the glucose moiety of sucrose. A rich source of RFOs are plants belonging to *Fabaceae* family and also by-products of e.g., the debittering process of alkaloid-rich lupin seeds or production of protein isolates from pea seeds (GULEWICZ 1988; FRĄCZEK *et al.* 2000).

Although RFOs perform a very important physiological function (LARSSON *et al.* 1993; HOR-BOWICZ and OBEJDORF 1994; GÓRECKI *et al.* 1997) from the nutritional point of view, RFOs are considered as antinutritional factors, because they are not hydrolyzed by mucosal enzymes in the small intestine of monogastric animals. They are fermented in the large intestine with liberation of gas causing arduous flatulence (CRISTOFARO *et*

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*al.* 1974; SAINI & GLADSTONES 1986; PRINCE *et al.* 1988).

During the last few years, studies on using FOs in chicken nutrition and production of so-called "health food" have been undertaken (BAILEY *et al.* 1991; WALDROUP *et al.* 1993). As a result of adding fructooligosaccharides to the fodder, a decrease of the amount of *Salmonella typhimurium* in the intestines of broiler chicken was observed. Similar results were obtained by CHOI *et al.* (1994) who additionally observed a decrease of pH in intestines caused by an increase of volatile fatty acids. *In vitro* studies showed the largest inhibitory effect on the growth of the most common Salmonella strains for *Bifidobacterium bifidum*, *Enterococcus faecium* and bacteria of *Lactobacillus* genus (OYZABAL & CONNE 1995).

The effect of  $\alpha$ -galactosides on the growth of bifidobacteria in intestines of chicken and the development of birds is unknown. On the other hand, there are numerous data suggesting that a group of these compounds may be a potential health ingredient of the diet as the prebiotics (GIBSON 1998; VAN LOO *et al.* 1999). The main aim of these studies was to assess the biological activity of different RFO preparations obtained from lupin seeds and their comparison with others. These studies were performed on a simple avian embryo model described by BEDNARCZYK *et al.* (1987).

## Material and Methods

### Reagents and materials

Seeds of lupin (*Lupinus albus* L.) cv. Multolupa were obtained from the Agricultural Research and Technology Development Service of the Agriculture and Commerce Council of the Junta de Extremadura (Spain). Fructooligosaccharides were given from Orafit S.A., Belgium; raffinose was supplied from Merck, Darmstadt, Germany; TPY medium was purchased by BTL Łódź, Poland.

### Preparation of RFOs

$\alpha$ -Galactoside preparations were isolated and purified from lupin seeds according to the method described by GULEWICZ *et al.* (2000). A slight modification of the method used by VILLALUENGA *et al.* (2004) was implemented for the preparation of low sucrose RFO.

### HPLC Analysis of RFOs

The analysis (separation and quantification) of two lupin RFO preparations was carried out by

high performance liquid chromatography HPLC-RI (FRIAS *et al.* 1994; GULEWICZ *et al.* 2002).

### Estimation of the time of injection and doses of oligosaccharides

For the determination of the optimal time of injection of the tested sugars, high sucrose RFO was applied to eggs on the first, eighth, twelfth and seventeenth day of incubation. The RFO preparation was injected with a dose 0.1763 mg/eggs in 200 ml of Ringer solution. Each experimental group contained twenty eggs. The assessment of the optimal time of injection was done on the basis of the number of bifidobacteria in feces of two-day old chicken.

The basic dose of oligosaccharides used in the experiment was 0.1763 mg/egg. In order to determine a range of the doses, the multiplicity (5x, 10x and 50x) of this dose were used. The evaluation of different doses of oligosaccharides was done on the basis of its effect on the body weight of nineteen day old embryos and chicken hatchability. Different doses (0.1763, 0.8815; 1.763 and 8.815 mg/egg) of high sucrose RFO were applied in the optimal time to four experimental groups containing forty embryonated eggs (of the same weight). Only Ringer solution was applied to the control group.

### Incubation and treatment

The experiment was conducted with Hybro G broiler breeder eggs from a commercial flock of hens. The eggs were incubated in a forced-air draft incubator equipped with an automatic egg turner (Pas-Reform) at a temperature of 37.8°C and 60 RH. On the 12th day of incubation the eggs were candled and those that were infertile or contained only dead embryos were removed. Then the eggs were divided into thirteen groups of forty eggs each, and a hole (0.8 mm) in the shell was made above the air cell.

The eggs of experimental groups (II, III, IV – low sucrose RFOs – RFO (L); V, VI, VII – raffinose – R; VIII, IX, X – fructooligosaccharides – FO; XI, XII, XIII – high sucrose RFOs – RFO (H)) were injected into the air cell with 0.2 ml of Ringer solution containing the following doses of oligosaccharides: 0.1763, 0.8815 and 1.763 mg/egg respectively. The doses of both RFOs were prepared on the basis of  $\alpha$ -galactoside content in the preparations. The hatchability of the treated embryos was registered.

### The influence of oligosaccharides on the growth of bifidobacteria

Fecal matter from 30 two-day old chicken of each group was collected into a tared test tube with 10 ml of sterile 0.85% sodium chloride solution

Table 1

Percentage content of particular sugars in lupin RFO preparations. \*Rha – rhamnose, Glc – glucose, F – fructose, Gal – galactose, Su – sucrose, I – inositol, R – raffinose, S – stachyose, V – verbascose

RFO	Rha*	Glc, F Gal	Su	I	R	S	V	Total RFOs	Total
(L)	0.91	0.13	2.10	-	10.50	59.50	24.52	94.52	97.66
(H)	4.02	3.13	20.57	1.84	10.60	45.32	13.92	60.84	99.40

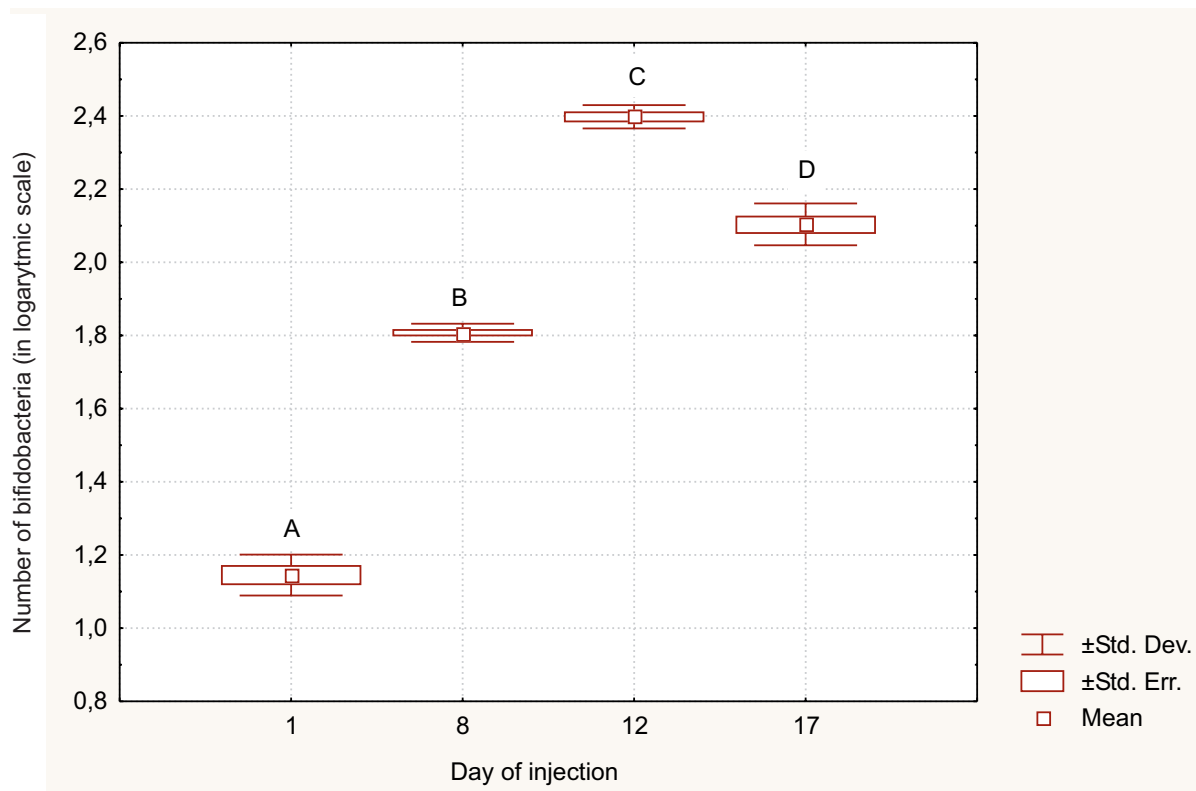


Fig. 1. Dependence between the day of RFO (H) injection into an egg and number of *Bifidobacteria* in feces of two-day old chicken. The results present the number of bifidobacteria in a  $10^{-7}$  dilution of one gram of feces and are expressed in logarithmic scale. Letters A-D following entries indicate statistical significance at  $P < 0.01$ .

and stored in a  $\text{CO}_2$  atmosphere. Fecal mass was determined. Decimal dilutions from  $10^{-6}$  up to  $10^{-9}$  were made from the suspensions. From each dilution, 1 ml was applied on a Petri dish covered with TPY medium – solid medium for the isolation of bifidobacteria (RADA & PETER 2001; RADA *et al.* 2001). The number of bifidobacteria was estimated on the TPY medium. Incubation was carried out in a  $\text{CO}_2$  rich atmosphere for 72h at  $37^\circ\text{C}$ . Results were calculated in colony forming units (CFU)/g of feces.

#### Statistical analysis

Statistical evaluation was made by analysis of variances with Tukey test for all groups. The dif-

ferences were considered significant at  $P < 0.05$  and  $P < 0.01$ .

#### Results and Discussion

The chemical composition of lupin  $\alpha$ -galactoside preparations are presented in Table 1. The major sugars in both lupin preparations are  $\alpha$ -galactosides (raffinose, stachyose, verbascose) – 94.52% for low sucrose content and 60.84% for the high sucrose one. In this pool, stachyose has the highest contribution, 62.95% and 74.5%, respectively. Both preparations differ in sucrose and other monosaccharide content. The RFO (H) preparation contains nearly ten times more sucrose (20.57%) in comparison to RFO (L). Besides su-

crose, RFO (H) contains more monosaccharides like rannose, glucose, fructose and galactose. In contrast to the low sucrose preparation, RFO (H) also contains inositol. The total content of  $\alpha$ -galactosides and other sugars in both preparations was at a level of 97.66% in RFO (L) and 99.4% in RFO (H).

Figure 1 presents the effect of RFO injection time on the number of bifidobacteria in feces of two-day old chicken. The injection time has a significant influence on the number of *Bifidobacteria* of two-day old chicken. A greater number of *Bifidobacteria* appeared in feces when the injection was done on the twelfth day. The statistical differ-

ences between particular injection times were on the level of  $P < 0.001$ .

The influence of different doses of the RFO (H) on the weight of nineteen-day old embryos is shown in Figure 2. The increase of body weight of embryos is proportional to the dose of the preparation from 0.0 to 1.763 mg/egg and the greatest increase of weight was noted at the dose of 1.763 mg/egg. At the dose of 8.815 mg/egg, a significant decrease of embryo weight was observed. Between the doses of 0.1763, 0.8815, 1.763 mg/egg and the dose of 8.815 mg/egg, statistical differences on the level of  $P < 0.01$  were observed. However, between the control group (dose 0.0) and the

Table 2

Influence of various oligosaccharides on mean number of bifidobacteria of two-day old chicken (CFU/g of fecal). \*Results are expressed as mean value standard deviation ( $\pm$ SD). Letters a-d following entries indicate statistical significance at  $P < 0.05$  and letters A-D at  $P < 0.01$ .

Dose [mg/egg]	C	RFO (L)	RFO (H)	R	FO
0.1763	$4.8 \times 10^7$ aA $\pm 29.82^*$	$4.8 \times 10^7$ aA $\pm 31.71$	$6.1 \times 10^8$ bB $\pm 393.15$	$2.1 \times 10^8$ aA $\pm 83.43$	$6.2 \times 10^8$ bB $\pm 365.26$
0.8815	$4.8 \times 10^7$ aA $\pm 29.82$	$1.4 \times 10^8$ aA $\pm 67.87$	$7.2 \times 10^8$ bcB $\pm 375.97$	$4.7 \times 10^8$ bB $\pm 254.42$	$8.8 \times 10^8$ cB $\pm 327.09$
1.763	$4.8 \times 10^7$ aA $\pm 29.82$	$7.8 \times 10^8$ bB $\pm 386.89$	$1.2 \times 10^9$ cdB $\pm 431.31$	$5.3 \times 10^8$ bB $\pm 281.44$	$1.1 \times 10^9$ cB $\pm 448.79$

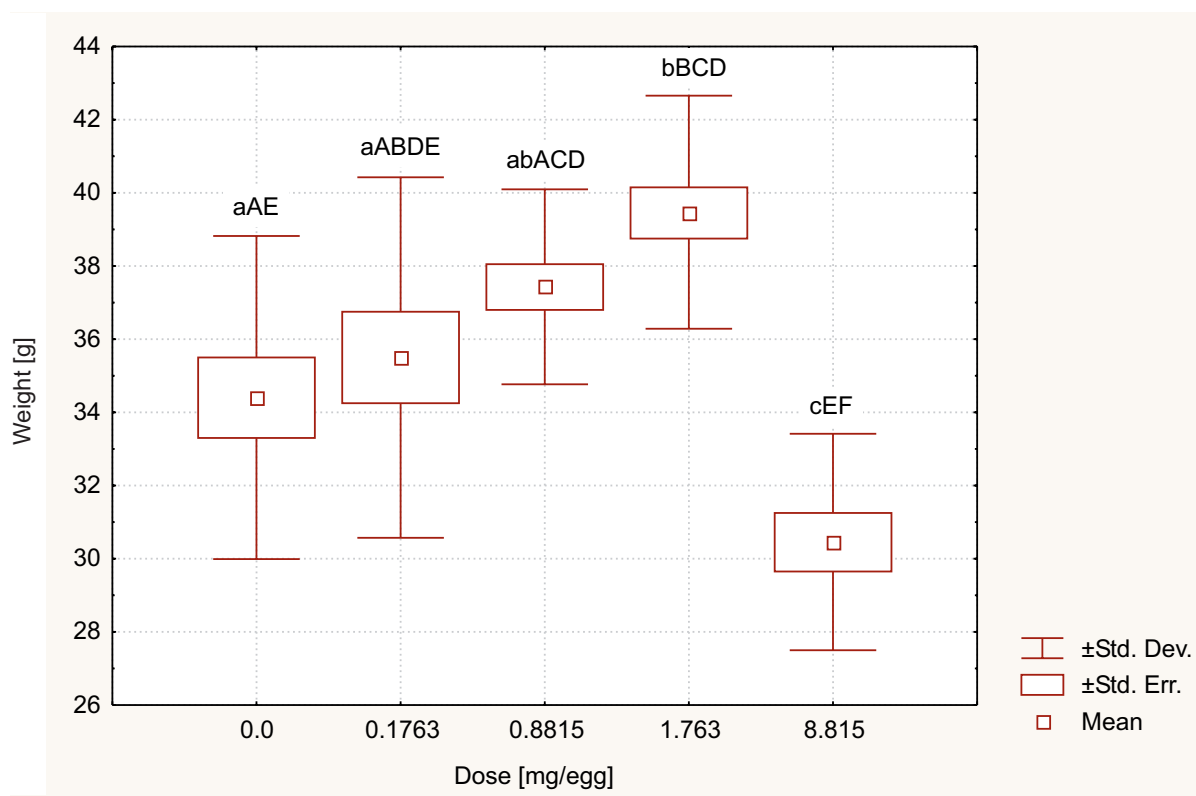


Fig. 2. Effect of the different doses of the RFO preparation on embryo weight. Results are expressed in logarithmic scale  $\pm$ SD and SE. Letters a-c and A-F following entries indicate statistical significance at  $P < 0.05$  and  $P < 0.01$  respectively.

Table 3

Dependence between doses of the preparations and number of bifidobacteria in feces (CFU/g). \*Results are expressed as mean value ± standard deviation (±SD). Letters a-b following entries indicate statistical significance at P<0.05 and letters A-B at P<0.01

Preparations	Doses [mg/egg]		
	0.1763	0.8815	1.763
RFO (L)	4.8 x 10 <sup>7</sup> ±31.71aA	1.4 x 10 <sup>8</sup> ±67.87bA	7.8 x 10 <sup>8</sup> ±386.89 cB
RFO (H)	6.1 x 10 <sup>8</sup> ±393.15aA	7.2 x 10 <sup>8</sup> ±375.97aA	1.2 x 10 <sup>9</sup> ±431.31bA
R	2.1 x 10 <sup>8</sup> ±83.43aA	4.7 x 10 <sup>8</sup> ±254.42bA	5.3 x 10 <sup>8</sup> ±281.44bA
FO	6.2 x 10 <sup>8</sup> ±365.26aA	8.8 x 10 <sup>8</sup> ±327.09bA	1.1 x 10 <sup>9</sup> ±448.79bA

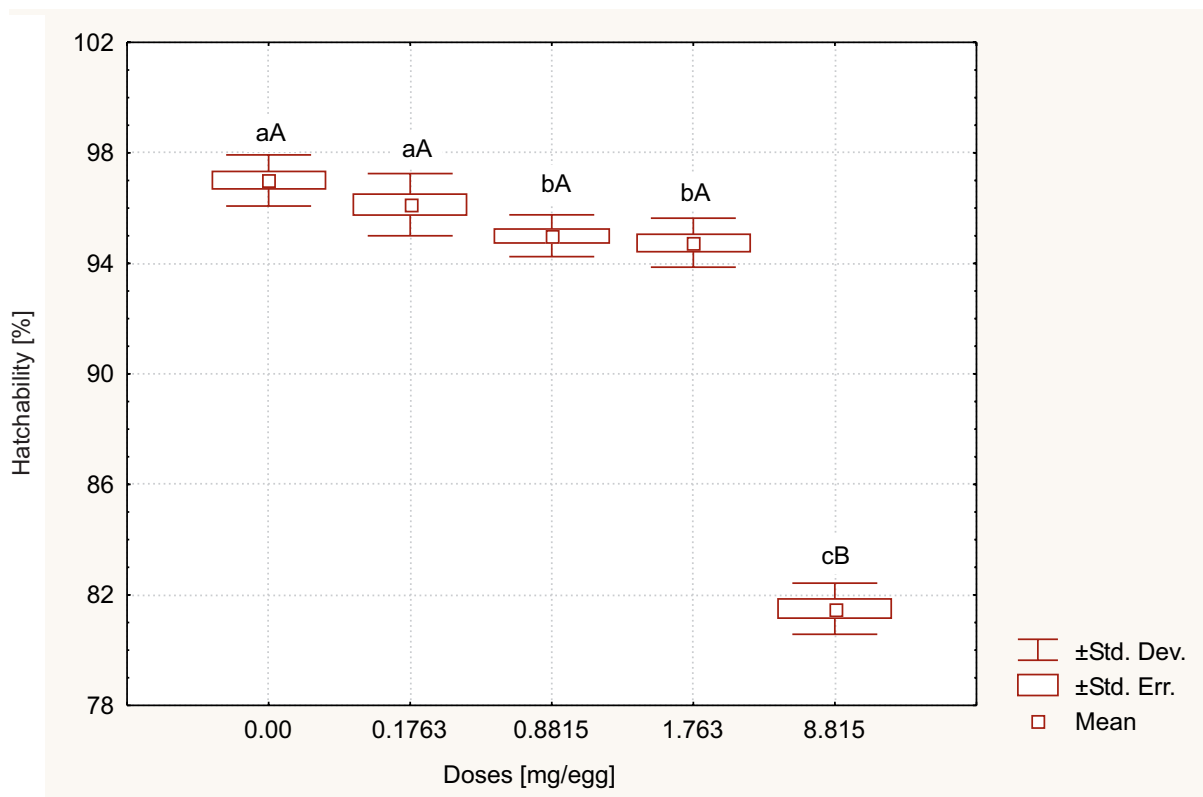


Fig. 3. Effect of the RFO preparation on chicken hatchability. Results are expressed in logarithmic scale ±SD and SE. Letters a-c and A-B following entries indicate statistical significance at P<0.05 and P<0.01 respectively.

greatest dose of preparation these differences were on the level of P<0.05.

Figure 3 presents the influence of different doses of RFO (H) on hatchability. The injection of doses of 0.8815 and 1.763 mg/egg caused a slight decrease of chicken hatchability. Between these doses and the control group (the dose of 0.0) and the dose of RFO (0.1763 mg/egg) statistical differences were found on the level of P<0.05. The injection of RFO at a dose of 8.815 mg/egg caused a distinct decrease of hatchability in relation to the above mentioned doses at P<0.01.

Table 2 illustrates the effect of various oligosaccharide preparations applied into eggs on the twelfth day of incubation, on the number of fecal *Bifidobacteria* of two days old chicken. The number of *Bifidobacteria* depends on the type of injected preparation and its dose. At the lowest dose (0.1763 mg/egg), the most evident increase of the number of *Bifidobacteria* in fecal for FO, RFO (H) and R was found. At the dose of the RFO (L), the number of *Bifidobacteria* was on the same level as in the control group. Similar results were noted when higher doses (0.8815 mg/egg) were applied, however, in this case a slight increase of the



number *Bifidobacteria* for RFO (L) was observed. The application of the highest dose (1.763 mg/egg) of oligosaccharides caused the most significant increase of *Bifidobacteria* for all experimental groups.

The relationship between the preparation dose and the number of *Bifidobacteria* in feces is shown in Table 3. Generally, for all experimental groups a linear growth increase of bifidobacteria populations exists after the administration of the all used doses of the oligosaccharides. This observation is especially true for the RFO (L) preparation which in the highest dose of 1.763 mg/egg is over 20 times more active in comparison to the initial dose of 0.1763. For other preparations an increase of oligosaccharide content caused approximately twice as high an increase of the bacteria in the final doses.

The beneficial effect of oligosaccharides, mainly FOs on monogastric organisms, is presently well documented (TOMOMATSU 1994; GIBSON 1998; VAN LOO *et al.* 1999). Relatively less is known about legume RFOs. Up to now, only soybean oligosaccharides have been an object of interest (JOHNSTON *et al.* 1971; TOMOMATSU 1994). Contrary to soybean, RFOs from other legumes contain verbascose and other oligosaccharides. The basic difference between FO and RFO is connected with the chemical composition and structure of these low molecular compounds. Fructooligosaccharides are a mixture of  $\beta(2-1)$  fructans with general structure  $GF_n$  or  $F_m$  in which: G – glucosyl unit; F – fructosyl unit; n, m – number of fructosyl units that are linked. RFOs are  $\alpha(1-6)$  galactosides linked to carbonic C-6 of the glucose moiety of sucrose.

The main aim of this paper was finding the correlation between chemical composition of oligosaccharides and their biological activity. The criterion used for the evaluation of biological activity of various oligosaccharides was their effect on chicken intestinal colonization by bifidobacteria. However, (i) the medium used was not selective enough for elimination of growth of other bacteria, (ii) not all bifidobacteria have the same ability of colonizing the alimentary canal. Further studies should be carried out on this topic.

The simplest method of determination of biological activity of various compounds and preparations are *in vitro* tests. However, the basic defect of these methods is simplifying and minimizing the number of variables influencing the studied properties. Hence, on the basis of the results of such tests, neither correct inference nor their relation to living organisms are possible, because it is impossible to take into consideration many coupled chemical reactions under *in vitro* conditions. Therefore, in order to obtain reliable information about the biological activity of preparations, *in vivo* tests are recommended.

A valuable model organism is the chicken as one of the primary models for embryology and development because its embryonic development occurs *in ovo*, making the embryo readily accessible. Avian eggs offer a mechanism for studying embryonic development and pathology in detail, because the chicken embryo is accessible from the period of gastrulation through neurulation and organogenesis until hatching. Moreover, the period of embryonic development of chicken comprises 21 days of incubation only, and as a result of it reproduction a large number of eggs, embryos or progeny are possible.

The different aspects of applications of *in ovo* technology in the poultry industry were presented by JOHNSTON *et al.* (1997) and RICKS *et al.* (2003). Moreover, the data of EDENS *et al.* (1997) indicate that the *in ovo* use of competitive exclusion (CE) agents is feasible for chickens. However, there are many pitfalls that await the use of *in ovo* application of CE agents, including the use of nonspecies-specific intestinal microbes and the use of harmful proteolytic, gas-producing and toxin-producing intestinal microbes.

Studies comparing the biological activity of various oligosaccharides using the chicken embryo model require the preliminary determination of such factors as time of injection and range of preparation dose.

As shown in Figure 1, the optimal time of injection on the twelfth day of incubation resulted in the largest number of bifidobacteria, while injection on the 1<sup>st</sup>, 8<sup>th</sup> and 17<sup>th</sup> days was significantly lower.

According to JOHNSTON *et al.* (1997), different factors influence effective delivery of *in ovo* injected substances to embryos. For example, the delivery is influenced by the chemical and physical features of the injected substances and by the site of injection in the egg, such as the embryo, the amnion, the allantois, the air cell, and the yolk sac. In case of prebiotics, the additional source of variation that has influenced the response to *in ovo* administration is the time from its injection to hatching, necessary to promote the growth of bifidobacteria. On the other hand, after twelve days of incubation, the completely developed and highly vascularized allantochorion serves as the more efficient transport route from air cell to the blood (ROMANOFF 1960).

The basic dose applied into the egg was 0.1763 mg that is equivalent of 3g of FO recommended for adult humans (TOMOMATSU 1994). As seen in Figures 2 and 3, the last dose had a clearly disadvantageous effect on both body weight of embryo and hatchability. The egg is a complete source of nutrients necessary for the developing embryo and it is known (WILSON 1997) that excesses just as

deficiencies of certain nutrients or egg constituents may affect embryo mortality. The present results indicated that the dose of 8.815 mg of oligosaccharides injected into the air cell is intolerated by the chicken embryo. For this reason, this dose was omitted in principal studies.

In spite of injection of various oligosaccharides in the same doses and under the same conditions, their effect on intestinal bacterial profile was quite different (Table 2 & 3). At the lowest dose of 0.1763 mg/egg, the biggest effect on the growth of *Bifidobacteria* was observed for FO and RFO (H). Surprisingly, at this dose the second RFO (L) – high purity preparation with low sucrose content showed activity on the level of control. The difference of biological activity between both preparations partially decay just at the 1.763 mg/egg dose, although still statistically significant. Similarly, pure raffinose at all doses showed the lowest activity in comparison to FO and RFO (H). As shown in Table 1, both RFO preparations differ in sucrose and monosaccharide content. In order to take into consideration the effect of these sugars (“contaminations”) on biological activity of both lupin preparations, all the applied doses were recalculated on total RFO content. Sucrose and monosaccharides present in the RFO (H) preparation caused an increase of its biological activity. Although this is difficult to explain, it may be suggested that these low molecular sugars (“contaminations”) create with RFO resistant complexes against microflora enzymes of the upper gut and, therefore, after entering the colon, may be an important additional carbon source for intestinal *Bifidobacterium*. This hypothesis is confirmed by the fact that sucrose injection to the eggs have not influenced the number of *Bifidobacteria* in the feces of two day old chicken (data not presented).

On the basis of the present studies it may be inferred that the chicken embryo is an excellent model for the investigation of biological activity of natural plant products and their effect on embryonic development. The results presented in this paper may be a very good base for further studies, e.g. concerning the way that oligosaccharide preparations applied during embryogenesis influence post-embryonic development of organisms (for example on meat trials of broiler), the effects of oligosaccharides on the intestinal profile of other bacteria including harmful and toxic ones, or oligosaccharides as substitutes of antibiotics injected during embryo development. Studies in these topics are presently being undertaken.

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