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Biotechnological researches concerning the multiplication of a *Lactobacillus plantarum* strain on media with pollen for the obtaining of a probiotic product

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Abstract

This study presents the multiplication of a Lactobacillus plantarum 2s strain on media containing pollen and honey. The next parameters were determined: CFU value, lactic acid production, glucide consumption and final product presentation. The experiments lasted 72 hours, with different percentages ground and unground pollen (P1 – 20g unground pollen, 3 g honey, 5 ml distilled water; P2 – 20g ground pollen, 3g honey, 5 ml distilled water; P3 – 20% unground pollen, 3% honey; P4 – 20% ground pollen, 3% honey), in flasks, at a temperature of 37^0C. The media were very well homogenized before inoculation. The inoculation took place only after the medium had acquired a homogeneous consistence. The inoculum represents a Lactobacillus plantarum 2s culture of 48 hours, on LE medium.

The tests concerning the nutritive value of the probiotic product were performed on Wistar rats, males and females, divided into lots of 10 animals. Every day probiotic product doses of 2 (lot 1), 20 (lot 2) and 20 mg/kg (lot 3) and a control lot, untreated were administered in the animals' food. During the four weeks of the experiment, there were no lethal cases with any of the lots and neither with the control lot. The animals were examined every day and did not show change in their appetite, behavior modification or clinical disease symptoms.

Keywords: *Lactobacillus plantarum*, pollen, honey, lactic acid, Wistar rats

Introduction

Probiotics are non-pathogen microorganisms, and, when ingested, have a positive effect on the host's physiology and health. They can influence gut physiology directly, but also indirectly by modulation of the endogen ecosystem or the immune system. Many observations were made, concerning probiotics, especially about their capacity to prevent and heal gut problems; though, only few probiotics strains proved efficient in controlled clinical cycles. [1, 2]

This finding made the search for new strains both possible and necessary, especially lactic-acid bacteria, in making food-medicine products, efficient in maintaining a good health, in improving the human gut medium with direct and indirect positive effects on the host. Though *Lactobacillus plantarum* is not a typical strain for human products, yet it is an important representative of lactic bacteria *Lactobacillus* gender. In the studies made by the Biotehnol researchers, a very good evolution of *Lactobacillus plantarum* strains on media with pollen and honey was proved. [3, 4]

A very important role also had the use of highly nutritive substrates for the microbial strain and for the host that consumes the final product. Pollen is a natural, nutritive source, very used in human therapy. Pollen and honey mixture is an important source for human health. This mixture is, at the same time, a favourable medium for the multiplication of bacteria producing lactic acid and it is used for the obtaining of products with a probiotic role. [5, 6]

An important factor in making such a product is the access of probiotic strain to the carbon source. A big problem is breaking the pollen grain and setting the nutritive substrate free in the fermentation medium. Medium hydration is very important. An optimum composition must be chosen, because a too high concentration of the carbon source in the medium can lead to a catabolic represion. From data in specialized literature, such a sold probiotic product must currently have as high viability as possible. [7, 8]

Thus, the final product must meet certain requirements for commercial presentation. Generally, it must have a homogeneous colour, composition and consistence. The taste must be close to that of pollen and honey, mixed with the traditional taste of products fermented with lactic-acid bacteria. Generally, the product must not contain unbroken pollen grains, but it must also be semisolid, with the pollen perfectly mixed with the honey. [9, 10]

Materials and methods

Biological material., A *Lactobacillus plantarum* 2s strain from the Biotehnol Center collection was used in the experiments.

Culture media and fermentation conditions. The used strain was kept at 4^0C on a medium containing 9% powder milk, 0,2% yeast extract (named LE by the authors). For revitalising the microorganism, 25 ml MRS sterile liquid is sown with 10% culture on LE. This takes place at 37^0C, in static conditions, for 24 hours.

For the researches, 4 media based on pollen and honey were used: P1 – 20g unground pollen, 3 g honey, 5 ml distilled water; P2 – 20g ground pollen, 3g honey, 5 ml distilled water; P3 – 20% unground pollen, 3% honey; P4 – 20% ground pollen, 3% honey. Pollen grinding for the P2 and P4 media was made a grinder at 15 second, 10 seconds break; the procedure was repeated 5 times. P1 and P2 are semisolid media achieved by mixture of the elements and a minimum quantity of water addition, enough to break pollen grain and to obtain a relatively homogeneous medium. The 4 media were inoculated with 10% *Lactobacillus plantarum* 2s strain developed on LE for 48 hours, at 37^0C.

The medium was prepared in flasks and, after inoculation, they were submitted to a temperature of 37^0 C. The flasks were maintained statically.

Glucides determination by o-toluidine method. The reducting glucides in the medium were determined by the o–toluidine method, made by the National Institute of Chemical-Pharmaceutical Research-Development – ICCF Bucharest.

Lactic acid determination. The acidity was determined by titration with sodium hydroxide 0,1N, following the correspondence: 1 ml NaOH 0,1N = 0,009008 g lactic acide.

Viability determination. For the establishment of the colonies formation units, the successive dilutions method was used.

Testing the nutritive value of the probiotic product. The nutritive value of the probiotic product was evaluated at a daily administration during 4 weeks. The experiments were performed on Wistar rats, males and females, divided in lots of 10 animals. The animals were

kept in vivarium conditions for aclimatisation, for a week, with standard food and ad libitum water. The animals chosen for the experiment did not present clinical disease symptoms and had the weight between 140 – 180 g.

There were four lots: three experimental lots with a daily food administration of probiotic product in doses of 2 (lot 1), 20 /lot 2) and 200 mg/kg (lot 3) and a control lot untreated. The animals were tested daily and weighed weekly.

At the beginning of the experiment and then every week the animals were weighed and, on the basis of the data obtained, the weight curve and the weight increasing coefficient were calculated. At the end of the experiment, the animals were sacrificed. Anatomo-pathological observations were made and blood samples were taken for biochemical determinations and the organs (liver, kidneys, milt**,** heart**,** adrenals**,** gonads) were weighed and then submitted to histo-pathological exams. The weight increasing coefficient was calculated according to the following formula:

$$\text{Increasing coefficient} = \frac{\text{weight increasing of the treated lot}}{\text{weight increasing of the control lot}} \times 100$$

Results and discussions

Lactobacillus plantarum 2s multiplication on media with pollen and honey

The strain multiplication was analyzed by making a comparison between two groups of culture media. The first group (P1 and P2 media) has a higher consistence because of the diminished water containing. The second group (P3 and P4 media) contains media similar to the usual composition. The determinations were made every 24 hours, due to the particular composition of the culture media.

At the beginning, the tests were performed on P1 and P2 media. The determination of lactic acid accumulation, viability and glucides consumption were made simultaneously. The tests lasted 72 hours and the probiotic product flavour and savour were also watched. Any probiotic product used in human nutrition must have certain organoleptic characteristics.

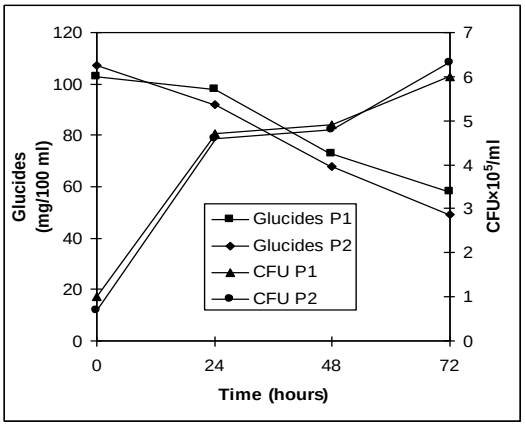

Figure 1. Glucides consumption and viability on P1 and P2

Figure 1 presents the consumption of glucides and the viability on the two media. P2 medium has a higher glucides concentration due to the ground pollen. In the first 24 hours a higher consumption was observed on this medium compared to P1, by 10 mg/100 ml. Between 24 hours to 48 hours the consumption is quantitatively equal, due to the fermentative action of *Lactobacillus plantarum* 2s strain. In exchange, over the last 24 hours of fermentation the difference is higher by 9 mg/100 ml on the P2 medium. Generally, the

culture medium requires a period of 24 hours for the strain to adjust. This period comprises a lag phase and an important period of the logarithmic growth phase. After that, the consumption has an important speed.

The viability on P2 after 24 hours does not reflect the higher glucides consumption, but during the next 24 hours, the microorganism multiplication exceeds the values obtained on P1 medium. After 24 hours, the viability values are approximately identical and they are not influenced by pollen status. Though the difference is not very high at 48 hours, it will prove essential, because after 72 hours the viability on P2 is higher than on P1, by $0,3 \times 10^5$ CFU /ml.

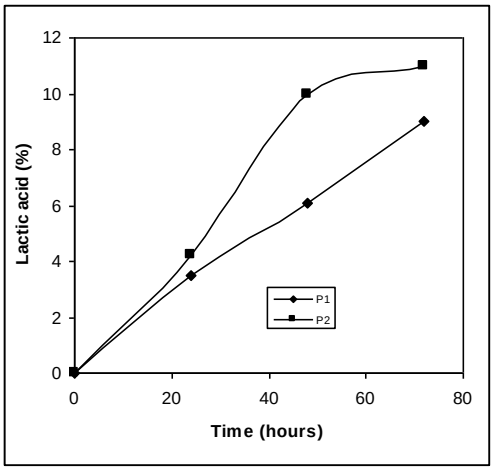

Figure 2. Lactic acid accumulation on P1 and P2

From **Figure 2**, it results that lactic acid accumulation depends on glucose consumption. A better accumulation is observed on P2 after 24 hours. The difference in the accumulation on the two media increases considerably after 48 hours, while after 72 hours, this difference is of 2%. The difference in decreasing over the last 24 hours may be explained by the fact that, in time, *Lactobacillus plantarum* strain adjusts to the medium based on unground pollen and after the pollen grains hydration, they break and the medium obtained is relatively identical to P2.

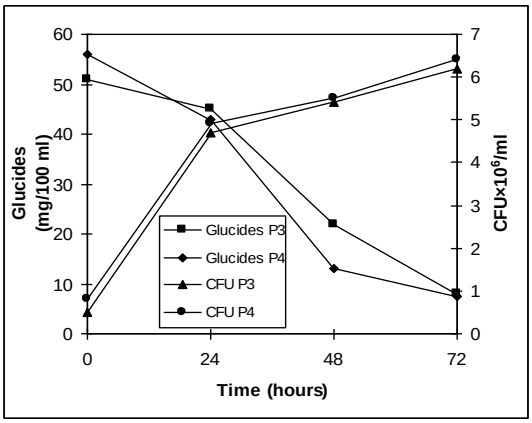

Figure 3. Glucides consumption and viability on P3 and P4

From **Figure 3** it results that a comparable hydration to the classic media influences positively microorganism development and stimulates glucides consumption. Identically to the media with low hydration, better results are obtained on P4, that contains ground pollen. In the first 24 hours the glucides difference between the 2 media is of 5 mg/100 ml. At 48 hours the difference increases considerably in the case of P4 medium. In exchange, at 72

hours the final glucides concentration is equal. This fact proves that for the current media pollen grains status does not have importance in the fermentative process. In the case of these two media it is not observed an adaptation period. After 24 hours the strain is in the logarithmic growth phase. This phase continues at the other periods due to the strain fermentative action.

Strain viability is maintained constant, thus, better results are obtained on P4. In the first 48 hours of fermentation, the viability on P4 is better, the difference being relatively constant, but until the end (at 72 hours), it seems that P4 offers better development conditions to *Lactobacillus plantarum* strain and therefore results by $0,2\times10^6$ CFU /ml better than on P3. Viability evolution is constant after 48 hours, due to the strain fermentative action.

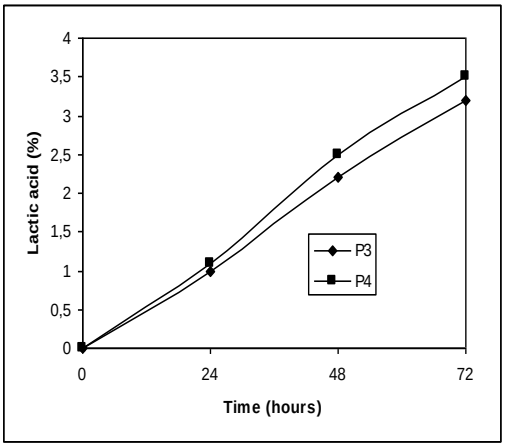

Figure 4. Lactic acid accumulation on P3 and P4

Figure 4 presents lactic acid accumulation on P3 and P4 media, better results being observed on the medium with ground pollen. It is very clear that the difference between the two media is maintained during the determination times. The lowest difference is observed at 24 hours, of 0,1%, while in the next 48 hours, the difference is constant, 0,3%. Though the difference between lactic acid accumulation and glucose consumption is not directly proportional, it results that higher glucose consumption assures a better strain viability on P4 medium.

Other characteristics pursued in the obtaining of the probiotic product are homogeneity and taste. The results are presented in **Table 1**.

From this taste, it results that homogeneity is kept during the fermentation period for P1 and P3 media. On the other two media, homogeneity is observed after 24 hours of fermentation and this fact is due to the fermentative activity of the lactic bacteria.

At the time of inoculation, the taste is sweet because of pollen and honey. Once the acid forms, the taste is an appropriate mixture of sweet and sour. Beside these characteristics, the product flavour was pursued; after 72 hours of fermentation, this flavour is characteristic to pollen and lactic acid.

Table 1. Several organoleptic characteristics of the probiotic product on the four culture media

Time (hours)	Product homogeneity				Taste			
	P1	P2	P3	P4	P1	P2	P3	P4
0	homogeneous	heterogeneous	homogeneous	heterogeneous	characteristic	characteristic	characteristic	characteristic
24	homogeneous	heterogeneous	homogeneous	heterogeneous	characteristic	sour sweet	characteristic	sour sweet
48	homogeneous	homogeneous	homogeneous	homogeneous	sweet and sour	sour sweet	sour sweet	sour sweet
72	homogeneous	homogeneous	homogeneous	homogeneous	sweet and sour	sour sweet	sour sweet	sour sweet

From a technological point of view, better results were obtained by using media containing a higher quantity of water, these media being close to those traditionally used in biotechnologies. Physical properties (humidity, grinding degree) of the raw material (pollen) have an important influence in obtaining a higher lactic acid quantity. This fact can be observed by evaluating the necessary times to obtain an optimum lactic acid quantity, by comparing process evolution after the data obtained on P1 and P3, on one hand and on P2 and P4, on the other hand.

Active microorganisms interact intensely with the environment by exchanging the necessary substances for the obtaining of metabolic products. Chemical composition of these products is very important for the microorganisms metabolic activities. Many investigations showed that bacteria in logarithmical phase are much more sensitive to the environmental stress than bacteria in stationary phase.

In experiments with starter organisms, it can be observed that the environment factors and bacterium signals at passing from logarithmical to stationary phase can have an important effect on the survival during stationary phase. Thus, stationary phase signal, considered to be carbon source lack, seems to be more propitious to survival than diminished pH in the case of a sufficient carbon source.

The nutritive value tests on the probiotic product

The weight evaluation of the rats treated with probiotic product, obtained on P1 medium, in doses of 2, 20 and 200 mg/kg is presented in **Figure 5** and **Figure 6**.

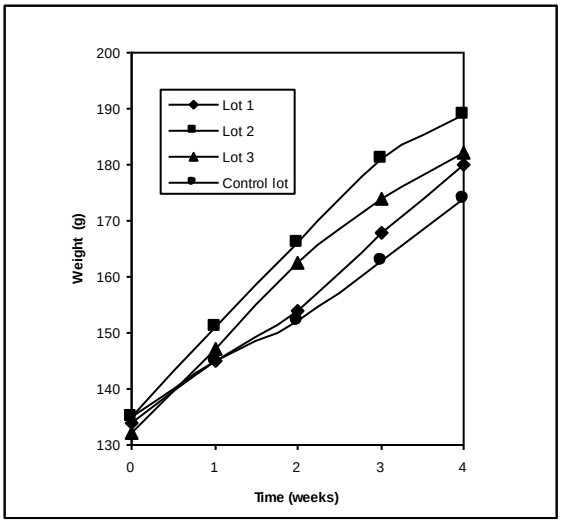

Figure 5. The weight evaluation of male rats treated with probiotic product

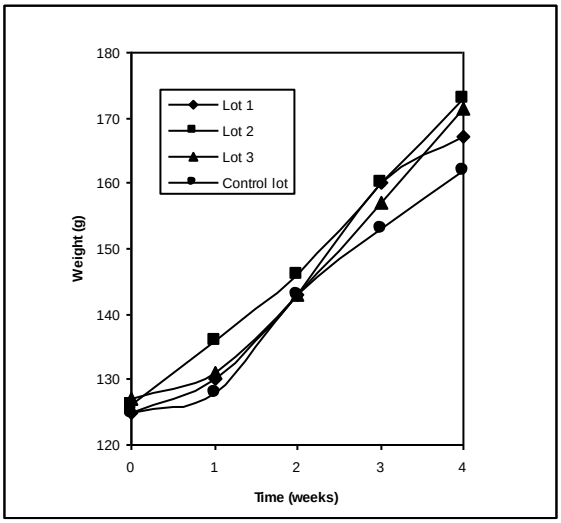

Figure 6. The weight evaluation of female rats treated with probiotic product

The values of weight increasing coefficient on male rats were: Lot 1 – 115%, Lot 2 – 130%, Lot 3 – 125%. The values of weight increasing coefficient on female rats were: Lot 1 – 115%, Lot 2 – 126%, Lot 3 – 118%. From the two figures, it results that the evolution of treated lots was similar to that of control lots on both genders, but after four weeks of administration, the weight coefficient of treated animals was superior to that of control lot animals, proving the high nutritive value of the tested product. The increasing coefficient were of 130% (on males) and of 126% (on females). The maximum weight increasing

coefficient was obtained at 20 mg/kg dose. It must be noticed that the maximum weight increasing coefficient was obtained in the second and the third week of experiment.

The media weights of the organs from the animals treated with the probiotic product for four weeks and also of the control samples lots are presented in **Table 2** and **Table 3**.

Table 2. Organs weights at male rats treated for 4 weeks with probiotic product

Organ	control lot	lot 1	lot 2	lot 3
Liver	5,568 ± 0,9137	5,463 ± 1,031	5,2723 ± 0,994	5,6171 ± 0,986
Milt	1,0985 ±0,1,854	1,0879 ± 0,2014	0,9623 ± 0,1967	0,9105 ±0,1765
Kidney	1,0137 ±0,1424	1,2657 ±0,2154	1,3298 ±0,1521	1,3603 ±0,1325
Heart	0,6618 ±0,0466	0,7174 ±0,0521	0,6852 ±0,04804	0,7084 ±0,04524
Adrenals	0,0798 ±0,0323	0,084 ±0,0214	0,090 ±0,095	0,089 ±0,039
Gonads	0,694 ±0,275	0,708 ±0,236	0,684 ±0,454	0,7184 ±0,3862

The internal organs did not present significant variations compared to the control lots and these variations are not correlated to the dose.

Table 3. Organs weights at female rats treated for 4 weeks with probiotic product

Organ	control lot	lot 1	lot 2	lot 3
Liver	5,3623 ±0,639	5,259 ± 0,84	5,340 ±0,4978	5,143 ±0,537
Milt	0,8781 ±0,214	0,8852 ± 0,135	0,908 ± 0,2031	0,7987 ±0,135
Kidney	1,136 ±0,239	1,129 ± 0,221	1,0812 ±0,178	1,0902 ±0,167
Heart	0,5907±0,0563	0,6163 ± 0,09	0,6126 ±0,062	0,599 ±0,0365
Adrenals	0,1103 ±0,0321	0,1039 ± 0,05	0,0953 ±0,036	0,1028 ±0,0530
Gonads	0,1598 ±0,0286	0,1648 ± 0,03	0,16736 ±0,0322	0,1624 ±0,0416

The female rats lots treated with probiotic product did not also present significant modifications of the internal organs weights compared to the control lots.

The values of biochemical parameters determined at the rats treated for 4 weeks with the probiotic product are presented in **Table 4** and **Table 5**.

Table 4. The values of certain biochemical parameters at male rats treated for 4 weeks with the probiotic product

Dose (mg/kg)	Glycemia (mg %)	Proteins (mg %)	Creatinine (mg %)	Urea (mg %)	Cholesterol (mg %)	Lipids (mg %)
200	124 ± 17	7,1 ± 0,5	0,644±0,05	37±6	33±6	301±12
20	126 ± 28	7,0±0,3	0,625±0,06	32±4	31±6	288±8
2	125 ± 19	6,9±0,6	0,629±0,07	35±7	29±4	303±38
-	121 ± 22	6,8±0,4	0,633±0,06	29±5	32±4	306±28

At the male and female rats treated for 4 weeks with probiotic product the determined biochemical parameters did not present significant variation at the treated lots compared to the control lot. This fact sustains the product toxicity lack at the liver and kidney level. The values of biochemical limits at all treated lots.

Table 5. The values of certain biochemical parameters at male rats treated for 4 weeks with the probiotic product

Dose (mg/kg)	Glycemia (mg %)	Proteins (mg %)	Creatinine (mg %)	Urea (mg %)	Cholesterol (mg %)	Lipids (mg %)
200	117±19	6,4±0,5	0,614±0,08	41±6	29±4	320±18

20	116±24	6,5±0,4	0,53±0,06	35±8	28,3±6	348±26
2	101±22	6,4±0,3	0,55±0,05	40±6	30,75±5	334±28
-	127±23	5,9±0,5	0,58±0,06	31±5	32±6	360±30

The anatomo-pathological exams performed at the end of the experiment did not reveal visible lesions of the internal organs. The histo-pathological exams revealed at the liver and the kidney little metabolic modifications at the 200 mg/kg dose. The other internal organs had comparable aspects to the control lot.

Conclusions

The positive effects of pollen on health are combined with microbial functional properties. Thus, the synergic effect of the components ensures the functionality of the product. The optimum pollen medium seems to be a medium containing water just as a classical one, P3 and P4, but very good results are obtained on P1 and P2. In this determination of the optimum medium, commercial elements of microbial strains were taken into consideration. A high viability at the media containing more water is very important, but the final decision must take in consideration more factors. In conclusion, media containing less water are optimum in the product obtaining, microorganisms viability having good values, though they are not very high. An important factor is the biosynthesized lactic acid quantity, because its presence is an additional guarantee of the probiotic product sterility. Pollen grinding in P2 and P4 seems to be essential for the final results.

Taste, flavour and homogeneity are important commercial factors. The product must have a taste and a flavour typical to the products containing bacteria producing lactic acid. They are very similar to the products available on the market containing such microorganisms. But all these are combined with pollen taste and flavour.

The probiotic product was tested for assessment of its nutritive value. The conclusion was that a daily administration for four weeks on lab animals determined a coefficient of weight increase as compared to the control lot. The maximum values of the coefficient of weight increase were obtained for the 20 mg/kg dose. The maximum variation of the weight increase was registered in the second and the third week of treatment. The analysis of the internal organs and the biochemical parameters did not reveal pathological modifications at the animals treated with probiotic product.

The combined effect of the microbial strain, with positive effects, is similar to the natural one, taking place in honeycombs, in beehives and to the effect of the lactic bacteria in the human organism.

Acknowledgements

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