The Antioxidant Effect of a Functional Product Based on Probiotic Biomass, Pollen and Honey

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Abstract

The effect of prebiotics on the multiplication of some probiotic strains used in a pollen and honey medium was investigated in laboratory conditions, aiming to obtain a bee bread-like product. The experiment were carried out using six different bacterial species belonging to the genera *Lactobacillus* (four strains) and *Bifidobacterium* (two strains). The prebiotics inulin, lactulose and raffinose were tested and compared to honey. A productivity similar to that of honey resulted for inulin and lactulose. The medium based on pollen and honey was supplemented with the two prebiotics, the optimal values being obtained by using ground pollen and inulin, in the case of viability, as well as in the case of total antioxidant activity. After seven days of fermentation, the viability was over 300 CFU×10^6/ g and the total antioxidant activity was over 45%.

Keywords: probiotic, inulin, lactulose, antioxidant

1. Introduction

The traditional and modern biotechnological processes have been used in order to obtain natural and functional food products, without alimentary additives. Functional products are obtained in a relatively small quantity, with natural flavours and perfumes. In the case of the other biotechnological products, the quantity, the aspect and price highly depend on the source of provenience of the raw material, the available equipment, their manufacture, the method used for manufacture and the final aspect of the product. In some cases, the traditional products use advantageous techniques which can determine both a natural aspect and a natural flavour. Such a product is that obtained from pollen, honey, strains of lactic bacteria and bifidobacteria. [1, 2, 3].

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Recent studies were focused on the possibility to obtain a product like the bee bread from the bees' hives that presents as a strong aliment of nutraceutical type. [4] Thus, its formation is relatively similar to that of the product we intend to obtain. Pollen is mixed with honey and, under the influence of anaerobic microorganisms (lactic bacteria), of humidity and of an approximate temperature of 35^0C, it transforms into bee bread. [5] The fermentation action of the aerobic and anaerobic microorganisms contained by the apiarian products is very important for its formation. The result, named bee bread, has high nutritive values. The bee bread has a high content of vitamins, minerals, amino-acids, and simple glucides, easy to assimilate. It is better tolerated by the human organism compared to the traditional pollen. It can be stored for long periods of time due to its content of lactic acid. The only clear interdiction is in the case of the persons suffering from diabetes, due to the content of easily to assimilate glucides. Moreover, the

persons with allergy to bee products should moderately consume it. [2, 3]

The object of the present work was to test the prebiotics in order to stimulate the multiplication of the probiotic strains used in the pollen and honey medium in order to obtain a bee bread-like product.

The effect of prebiotics was compared to honey, which is known as natural prebiotic stimulant mainly for *Bifidobacterium* strains. The aim of this study was to obtain a value similar to that accepted for products which contain such microorganisms ($CFU \times 10^6$/g product). The tests were carried out without additives admixtures in order to maintain the aspect of the product.

2. Materials and methods

Microorganisms and culture media. The probiotic strains *Lactococcus fermentum* BS2, *Lactobacillus plantarum* BS1, *Lactobacillus plantarum* BS3, *Lactobacillus paracasei* BS6, *Bifidobacterium bifidum* BS4, *Bifidobacterium bifidum* BS5 belonging to the Collection of the Faculty of Biotechnology of our University were used. These strains were kept in the freezer, at a -82^{o}C temperature, on a protective medium containing 20% glycerol and then revitalized on a MRS for the strains of *Lactobacillus* and MRS+0,2% cysteine hydrochloride, for *Bifidobacterium* strains.

The following culture medium was used in order to obtain biomass: glucose 5%, yeast extract 0.5%, peptone 1%, meat extract 0.5%. 1 ml/l nutritive solution with the following content (g/l): $MgSO_4$ 100g, $MnSO_4$ 50g, biotin 2g, NH_4Cl 30g, K_2HPO_4 26 g, KH_2PO_4 40g was also added in the medium. The biomass obtained was separated by centrifuging with a *Hettich* cooling centrifugal separator. Then the biomass was lyophilized in an *Alpha 1 – 2D* freeze-dryer. The inoculation was performed with 10% lyophilized culture.

In order to choose the prebiotic, MRS/MRS+0.2% cysteine hydrochloride (Cys) was supplemented with 1% inulin, lactulose and raffinose. The following parameters were observed: the growth curve by using Biospektrum and the synthesis of lactic acid in order to choose the prebiotic.

With the aim of testing the mixture of prebiotic-pollen –honey, the following media were used: B1: 20g unground pollen, 5g honey, 5ml distillated water; B2: 20g ground pollen, 5g honey, 5ml distillated water. They were supplemented with 1% inulin. B3, B4 media are supplemented with 1% lactulose, instead of inulin.

Inoculation was performed with 10% probiotic lyophilized biomass. The proportion between the lyophilized biomasses was as follows: 10% *Lactococcus fermentum* BS2, 15% *Lactobacillus plantarum* BS1, 15% *Lactobacillus plantarum* BS3, 10% *Lactobacillus paracasei* BS6, 25% *Bifidobacterium bifidum* BS4, 25% *Bifidobacterium bifidum* BS5. Pollen grinding was performed by means of a laboratory mill in successive stages, 15 seconds each, with 10s breaks, operation that was repeated 5 times. The medium was separated in plastic airtight recipients who were introduced, after the inoculation, at a 37^{0}C temperature. The tubes were statically maintained.

Determination of the glucides quantity by o-toluidine method. It was accomplished by means of the o-toluidine test, made by the National Institute for Chemical and Pharmaceutical Research and 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. [6]

Viability determination. The method of successive dilutions, using the medium of MRS/MRS+0,2% cysteine hydrochloride and supplemented with 0,1% acetic acid, 0,01% sorbic acid and 0,01% sodium nitrate in order to inhibit the accompanying flora was used to establish the number of CFU/g product. It was used for ColonyQuant analysis.

Determination of the total antioxidant activity. The antioxidant activity was measured by the determination of the linking capacity of the free radical 1, 1 – diphenyl – 2 – picrilhidrazil (DPPH). 50 µl of extract were mixed with 5ml ethanol solution DPPH 0,004%. After 30 minutes, the absorbance at 517 nm was read. [7]

Statistical analysis. The experimental results are expressed as mean ± standard deviation of triplicate measurements. Differences are considered significant when $p<0.05$. [8]

3. Results and discussion

In our first experiment we have achieved the determination of the best prebiotic (inulin,

lactulose, raffinose) for the stimulation of each separate microbial strain. The effect of supplementing the medium MRS/ MRS+0.2% cysteine hydrochloride with prebiotic for each strain was determined separately. These researches are made in parallel with using honey as probe, because it has an effect similar to that of the prebiotics. It directly stimulates the probiotic strains of lactic bacteria and bifidobacteria strains.

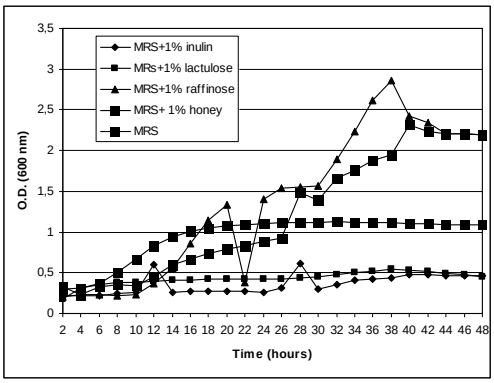

Figure 1. The evolution of *Lactobacillus plantarum* BS1 in the presence of the prebiotics

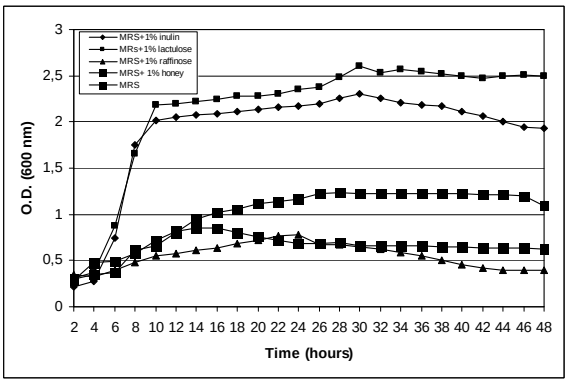

Figure 2. The evolution of *Lactobacillus fermentum* BS2 in the presence of the prebiotics

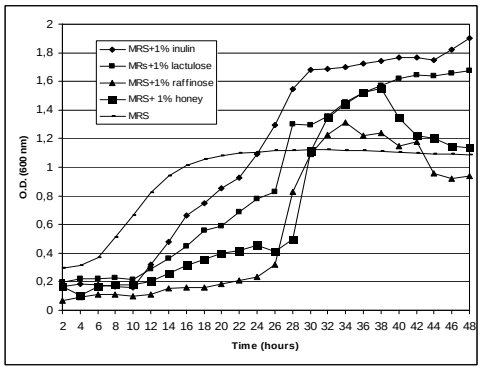

Figure 3. The evolution of *Lactobacillus plantarum* BS3 in the presence of the prebiotics

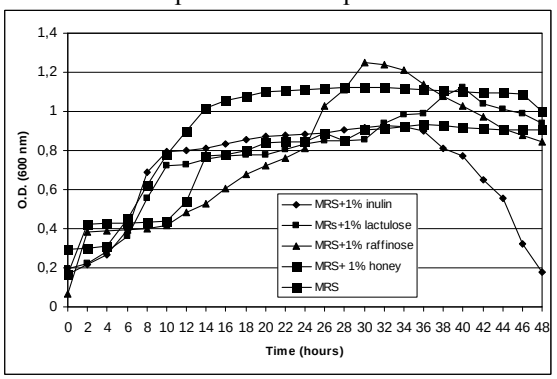

Figure 4. The evolution of *Lactobacillus paracasei* BS6 in the presence of the prebiotics

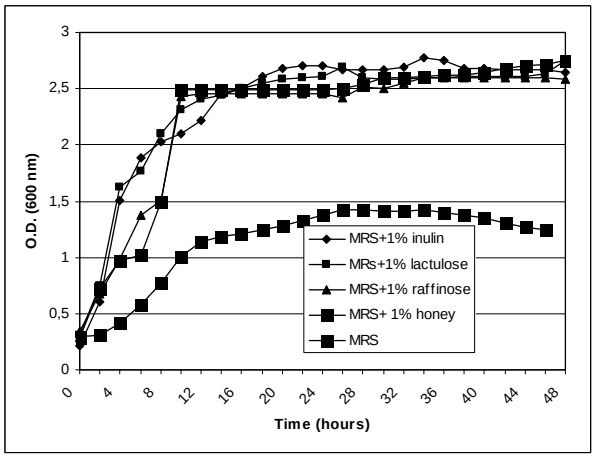

Figure 5. The evolution of *Bifidobacterium bifidum BS4* and *Bifidobacterium bifidum BS5* in the presence of the prebiotics

From the figures (**Figure 1** – **Figure 5**) presented, it resulted that the best results were obtained for inulin and lactulose. The supplementation with 1% of the honey medium determined the obtaining of some maximal values for all these strains. Maximal values were obtained for raffinose in the case of *Lactobacillus plantarum* BS1 and *Lactobacillus paracasei* BS6 strains. The average viability for MRS supplemented with inulin is 7×10^9 CFU/ml

and 9×10^8 CFU/ml for MRS supplemented with lactulose. Raffinose determines an average viability of only 6×10^6 CFU/ml, similar to that of MRS (4×10^6CFU/ml). The usage of inulin and lactulose determined a longer logarithmical phase of growth, developed in time. The strains entered the decline phase after approximately 40 hours of fermentation. It should be noticed the evolution of *Bifidobacterium bifidum* BS4 and *Bifidobacterium bifidum* BS5 strains which had a maximum evolution in the case of all prebiotics, as well as for honey. A maximal value can be noticed only for inulin, after 36 – 38 hours of fermentation.

These findings also resulted from the correlation of data regarding the growth curves of the strains to those of the lactic acid synthesis, as shown in **Table 1**. Besides the need to obtain a quantity as great as possible of probiotic bacteria biomass, the aim was also to keep the sterility of the growing medium. The synthesis of lactic acid represented a natural and very efficient method to avoid the possible undesired contaminations.

As shown in **Table 1**, the most productive strain was *Lactobacillus fermentum* BS2. The weakest strain from the point of view of lactic acid synthesis was *Lactobacillus plantarum* BS1. Comparing the synthesized quantity and the periods during which this quantity was at the maximum level, it resulted that the quantities globally obtained were the most reduced in the case of using raffinose. For the two strains of *Bifidobacterium*, a maximum value resulted in the case of honey, fact that was also certified by many previous researches. For the other prebiotics, the maximum value was synthesized within 24 hours and then values of at least 0.4% were obtained for raffinose. Anyhow, the value was approximately 40% of that obtained for honey.

As shown in **Figure 6,** B2 medium, that contained inulin, determined the synthesis of the maximum quantity of lactic acid, fact that was important for the obtaining of a competitive product. For B1medium, the synthesis of lactic acid attained a maximum value within 3 days of fermentation, but the smallest quantity was obtained in the end.

Table 1. The values of lactic acid after the supplementation with prebiotics

Strain	Prebiotic	Lactic acid (%)			
		12 hours	24 hours	36 hours	48 hours
Lactobacillus plantarum BS1	MRS	0,038	0,08	0,11	0,08
	Inulin	0,042	0,091	0,13	0,082
	Lactulose	0,007	0,011	0,0068	0,0005
	Raffinose	0,031	0,056	0,01	0,0024
	Honey	0,033	0,04	0,033	0,035
Lactobacillus fermentum BS2	MRS	1,5	2,0	1,55	1,5
	Inulin	1,74	2,89	1,96	1,9
	Lactulose	2,08	2,97	1,96	1,3
	Raffinose	0,177	0,362	0,28	0,2
	Honey	0,255	0,86	1,24	1,01
Lactobacillus plantarum BS3	MRS	0,05	0,1	0,2	0,21
	Inulin	0,054	0,101	0,211	0,243
	Lactulose	0,071	0,123	0,86	1,11
	Raffinose	0,142	0,204	0,266	0,33
	Honey	0,081	0,172	1,12	1,05
Lactobacillus paracasei BS6	MRS	1,0	2,0	1,0	1,52
	Inulin	1,2	2,2	1,05	1,62
	Lactulose	1,4	1,04	2,08	0,81
	Raffinose	1,41	0,66	0,55	0,3
	Honey	1,1	1,25	1,11	0,66
Bifidobacterium bifidum BS4 and *Bifidobacterium bifidum* BS5	MRS	1,0	0,7	0,21	0,2
	Inulin	1,1	0,74	0,3	0,24
	Lactulose	1,55	0,67	0,3	0,2
	Raffinose	0,78	0,62	0,46	0,38
	Honey	1,4	1,05	0,79	0,74

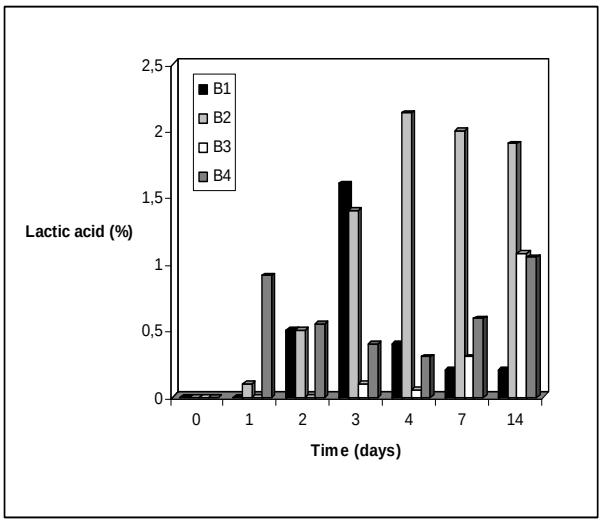

Figure 6. Quantity of lactic acid of probiotic strains (BS1, BS2, BS3, BS4&BS5, BS6) on B1, B2, B3, B4 media

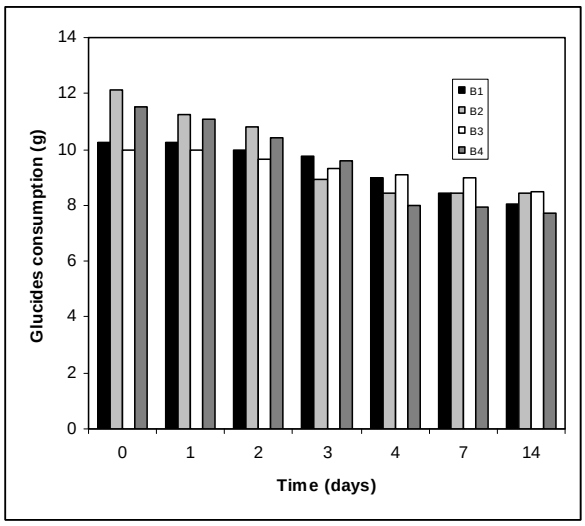

Figure 7. Glucides consumption of probiotic strains (BS1, BS2, BS3, BS4&BS5, BS6) on B1, B2, B3, B4 media

This finding was mainly determined by the fact that the pollen is not ground and, in this situation, the nutritive substances were discharged due to the fermentative action of the microorganism. Such a situation was found in the case of B3 and B4 media which used lactulose. For B3, that contained unground pollen, the release of pollen due to the fermentative action of microorganisms determined the obtaining of a supplementary quantity of lactic acid such as in the case of B4 medium in the 14th day. It can be even noticed in the figure a supplementary quantity, which did not have a major importance for obtaining the desired result.

If **Figure 6** and **Figure 7** are coordinated, it can be noticed that the synthesis of lactic acid developed concomitantly with that of the general consumption of glucides. It can be also noticed an accentuated decrease of glucides where an important synthesis of lactic acid developed. Generally, the decrease was not significant from the quantitative point of view, because, due to the fermentation process, the unground pollen mainly determined the gradual release of glucides in the medium.

As shown in **Figure 7,** the strains developed on the four media, synthesized the lactic acid and consumed a part of the reducing glucides. The maximum value obtained was found on B4 medium, which contained lactulose.

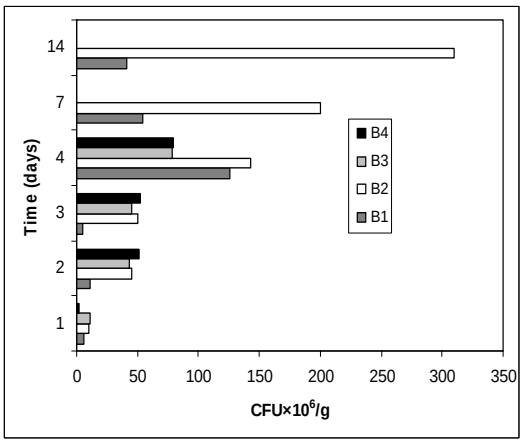

Figure 8. The viability of probiotic strains (BS1, BS2, BS3, BS4&BS5, BS6) on B1, B2, B3, B4 media

On the contrary, B3 that also contained lactulose presented the smallest consumption , but this medium also contained unground pollen. Although the glucides consumption after the supplementation with inulin was quicker than the consumption in B3 and B4 media, relatively equal values were obtained in the end.

The fact that B2 was the optimal medium was also certified by the diagram of viability (**Figure 8**). The results were similar from the point of view of the tendency with those obtained during the determination of lactic acid. Another significant aspect in this situation is that all the results were obtained for a minimum CFU×10^6. It was used as a minimum dilution in order to validate the medium formulas.

As it is not sufficient that the functional products we use have only some of the necessary properties,

in this situation, the aim was to find some supplementary properties which were to make the product more competitive. Consequently, it was aimed at the determination of the antioxidant capacity, an important feature for such products. The antioxidant activity that is presented in **Figure 9** was determined at the end of the 14 days of fermentation for each medium (B1, B2, B3 and B4).

As shown in **Figure 9**, the maximal antioxidant activity was determined for B1 and B2 media. The difference between them was very small, both for 7 as well as for 14 days. The maximum value was noticed in the case of B2 medium. Moreover, a decrease of the total antioxidant activity gradually occurred, but the level still remained very high, above to that obtained by using vitamin E (41% at 1 mg/ml vitamin E). [9]

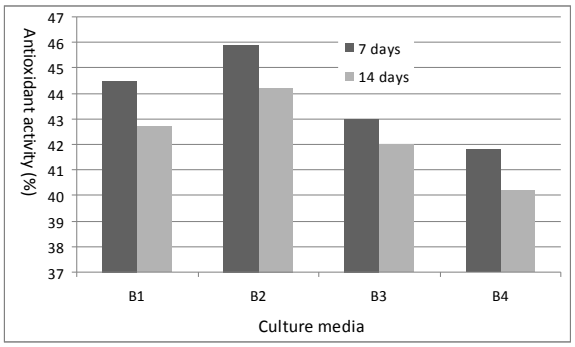

Figure 9. Total antioxidant activity after 7 days and 14 days of fermentation

It should be also noticed that the maximum time of incubation was 14 days. After this interval, it could be ascertained a darkening of the product color, thus resulting a non-commercial aspect. In the case of B1 and B3 variants, a zone with water in reduced quantity appeared during the incubation. For the other two variants, the aspect was homogeneous during the whole period. At the end, in the case of B1 and B3 variants, the color was darker unlike B2 and B4 cases. B1 and B3 developed a thin crust at the surface, under which there was a relatively non-homogeneous composition.

4. Conclusions

In conclusion, inulin and lactulose determined the obtaining of the optimal results among the tested prebiotics.

This fact reflected upon the quantity of biomass obtained, as well as upon the quantity of lactic acid. From the point of view of the evolution in the case of pollen and honey media, the grind of the pollen directly influenced upon the evolution of the strains used. The best evolution was registered in the case of variant B2. This version contained inulin as added prebiotic. This finding was noticed in the case of viability, the difference at 14 days among version B2 and the other three versions being significant, as well as in the case of total antioxidant activity.

Acknowledgements

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