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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (4): 613-617, 2008
ISSN 1028-8880

Obtaining of a Symbiotic Product Based on Lactic Bacteria, Pollen and Honey

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Abstract: This study presents the evolution of a *Lactobacillus plantarum* 2s strain and of a *Lactobacillus acidophilus* 1a strain on media containing pollen and honey. The following parameters were determined: CFU value, lactic acid production, sugar consumption and presentation of the final product. The researches were performed during 72 h, in tightly closed recipients, at a temperature of 37°C, containing various quantities of milled or non-milled pollen (P1: 20 g non-milled pollen, 3 g honey, 5 mL distilled water; P2: 20 g milled pollen, 3 g honey, 5 mL distilled water; P3: 20% non-milled pollen, 3% honey; P4: 20% milled pollen, 3% honey). The media were very well homogenized before inoculation. The inoculation was made only after the medium gained a homogenous consistency. The inoculum consists of a 48 h culture of *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus*, on LE medium, in equal proportions. The testing of the nutritive value of the symbiotic product was made on wistar rats, males and females, divided into lots of 10 animals each. The animals were administered symbiotic product every day, in their food, in intakes of 2 mg kg^{-1} (lot I), 20 mg kg^{-1} (lot II) and 200 mg kg^{-1} (lot III), compared to a control. During the 4 weeks of the experiment, no lethality cases were recorded in any of lots, or in control. The animals involved in the experiment were examined daily and did not present changes of appetite, of behavior or clinical signs of disease.

Key words: *Lactobacillus*, pollen, honey, cholesterol, wistar rats

INTRODUCTION

Probiotics are non-pathogenic microorganisms and when they are ingested they exercise a positive effect on the host's health and physiology. They can influence the physiology of the gut both directly and indirectly by modulating the endogenous ecosystem or the immune system (Reid *et al*., 2003). There are many references regarding probiotics, particularly to their potential in preventing or healing gut problems; however, only several strains of probiotics have proven to be efficient in the controlled clinical cycles (Fernandes and Shahani, 1990).

Among the microbial strains considered to be probiotics, the following can be itemized: *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Streptococcus thermophilus*, *Bifidobacterium bifidum* (Lee *et al*., 1999). A growing emphasis has been recently laid on the use of the *Lactobacillus plantarum* strain, as a human use symbiotic product. Originally used as an animal use probiotic strain, it is now proven that, in humans, it has a more and more important effect in cholesterol assimilation. Because of this reason, the *Lactobacillus plantarum* strains are more and more often used in the attempt to support the elimination of the cholesterol in hypercholesterolemia patients (María *et al*., 2005).

Another issue recently approached in the study of symbiotic products is that of gut problems. Although there are some studies and even probiotic products against colitis, lately the emphasis has been laid on preventing the colon cancer formation by using symbiotic products (Meydani and Woel, 2000). If by now the emphasis used to be laid on just one probiotic strain (e.g., *Lactobacillus acidophilus* or *Streptococcus thermophilus*), the last studies show the use of a product that contains two or several probiotic strains and a

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probiotic product i.e., a symbiotic product. The role of the probiotic is to stimulate, at the gut level, the development of probiotic strains of the *Lactobacillus* and *Bifidobacterium* type (Overton *et al*., 1994).

Special emphasis is laid on these two issues, because they appear because of environment pollution, sedentariness, consumption of food additives, high consumption of fats and low-fiber content food (Reid, 1999). This way, it is considered that the change of the food diet leads to the decrease of the risk of colon cancer formation, by ingesting fibers, prebiotic and symbiotic products. The factors that lead to the formation of colon cancer are inhibited by the consumption of symbiotic products, as these products inhibit the precancerous lesions and even tumors (Roberfroid and Delzenne, 1998).

Honey has lately been very often quoted as a prebiotic product. In its composition there are some fructo-oligosaccharides that are considered to be prebiotic. The combination between pollen and honey is an ideal medium, full of nutritive substances, for the multiplication of these types of bacteria. The final product offers, in exchange, a very good protection of the probiotic strains, due to its composition. This finding is important because probiotics are inhibited during the intestinal transit. Thus, the mixture of pollen with honey provides an important protection to microbial probiotic strains (Salminen *et al*., 2004).

The objectives of this study were the following: (1) Determination of the pollen and bee honey on growth and development and the effect of the bee honey on growth, development and activity of the *Lactobacillus plantarum* and *Lactobacillus acidophilus* strains, (2) The bio-productive effect on the tested lab animals and (3) Influence of the symbiotic product on the cholesterol level of the tested lab animals.

MATERIALS AND METHODS

The first objective was studied in the microbial bio-technology labs of the Bio-technologies Faculty and Biotehnol Center and the 2nd and 3rd objectives were studied in the pharmacy-bio-technology labs of the National Institute for Chemical Pharmaceutical Researches-ICCF Bucharest.

Biological material: In the research, the *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains were used, existing in the collection of the Biotehnol Center.

Culture media and fermentation conditions: The strains used were kept at the temperature of 4°C on a medium containing 9% milk powder and 0.2% yeast extract (called LE by the authors). To revitalize the microorganism, a volume of sterile MRS liquid of 25 mL is sowed with 10% culture on LE. It takes place at 37°C, under static conditions, for 24 h (Vamanu *et al*., 2004).

To perform the research, 4 pollen- and honey-based media were used: P1: 20 g non-milled pollen, 3 g honey, 5 mL distilled water; P2: 20 g milled pollen, 3 g honey, 5 mL distilled water; P3: 20% non-milled pollen, 3% honey; P4: 20% milled pollen, 3% honey. The media P1 and P2 are semisolid media obtained by mixing the components and adding a minimum quantity of water, but sufficient to smash the pollen grains and obtain a relatively homogenous medium. The 4 culture media were inoculated in a ratio of 1% with the *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains, developed on LE, 48 h, at 37°C (Vamanu *et al*., 2004).

The medium was prepared in tightly closed plastic recipients and after inoculation they were placed at the temperature of 37°C. The tubes were statically maintained.

Determination of the sugar quantity by using the o-toluidine method: It was performed with the o-toluidine test, made by the National Institute of Chemical-Pharmaceutical Research-Development-ICCF Bucharest (Roşoiu *et al*., 2005).

Determination of the lactic acid quantity: The lactic acid accumulation was determined by titration with HCl 0.1N. For determination, the fact that 1 mL HCl 0.1N corresponds to 0.009008 g lactic acid is taken into account (Roşoiu *et al*., 2005).

Determination of the viability: To establish the number of Colony Forming Units (CFU) the successive dilutions method was used. For determination, the Rogosa medium was used (Haddadin *et al*., 2004).

Testing of the nutritive value of the symbiotic product: The experiment was made on Wistar rats, males and females, divided into lots of 10 animals each. The animals were kept in the acclimatization vivarium conditions for one week, with standard food and *ad libitum* water. For the experiment, the animals that showed no clinical signs of disease and whose body weights were somewhere between 140-180 g were chosen.

Four lots were formed: three experimental lots, which were administered symbiotic product every day, in their food, in intakes of 2 mg kg^{-1} (lot I), 20 mg kg^{-1} (lot II) and 200 mg kg^{-1} (lot III) and a control. The animals were monitored every day and weighed every week.

In the beginning of the experiment and then every week, the animals were weighed, calculating the weight gain index. Blood samples were taken to determine the cholesterol level.

The weight gain indexes in the 3 batches were calculated by the following formula:

$$\text{Gain index} = \frac{\text{Weight gain of lot}}{\text{Weight gain of control}} \times 100 \text{ (Vamanu } et\ al.\text{, 2006)}$$

RESULTS

Development of the Lactobacillus plantarum 2s and *Lactobacillus acidophilus* 1a strains on pollen and honey-based media: The development of the strains was analyzed comparatively on the two groups of culture media. The first group (P1 and P2 media) has a higher consistency due to the low water content. The second group (P3 and P4 media) contains the media similar to the classic ones.

The determination of the lactic acid accumulation, the viability and the sugar consumption were made in parallel. The development period is of 72 h, also monitoring the aroma and savor of the symbiotic product.

First of all, the quantity of lactic acid was determined, because this is an important feature in such a product. The existence of a quantity as large as possible is an additional guarantee for the preservation of the product sterility. The largest quantity is found in the P2 medium (Fig. 1). For the P1 medium, the non-milled pollen determines a weaker synthesis of the lactic acid, which is due to a large quantity of nutrients located in the pollen grain, which are gradually released due to the fermentative action of the 2 strains.

For the classic media, the largest accumulation is on the P4 medium, representing 50% of the quantity synthesized on the P2 medium. The low quantity of nutrients, due to the large quantity of water, determines low values of the lactic acid.

The largest sugar consumption is on the milled pollen media, P2 and P4. From the viewpoint of the quantity remained after 72 h of fermentation, the strains consume most of the sugar existing on the P3 and P4 media. But, from the viewpoint of the consumed quantity, the P2 medium is optimal because, when using it, the strains use the largest quantity of sugar (Fig. 2).

Figure 3 shows that the P2 medium is optimal for the concomitant cultivation of the *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains, with a view to obtain a symbiotic product. Even if a large viability is also obtained on the P3 and P4 media, the large quantity of the water existing in them needs additional processing.

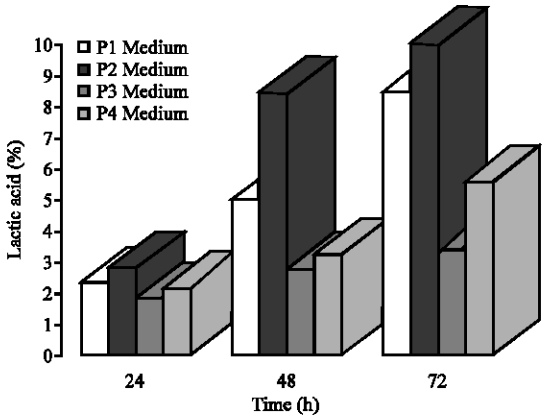

Fig. 1: Production of lactic acid on the P1, P2, P3, P4 media

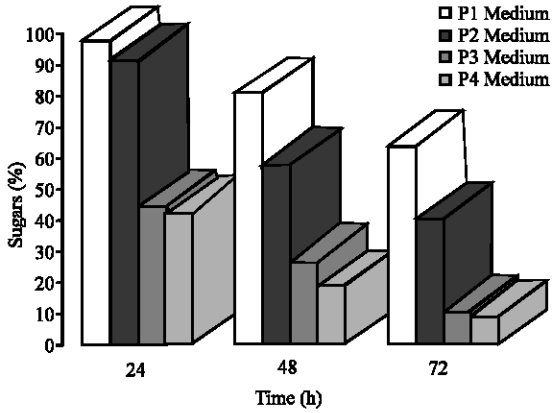

Fig. 2: Sugar consumption on the P1, P2, P3, P4 media

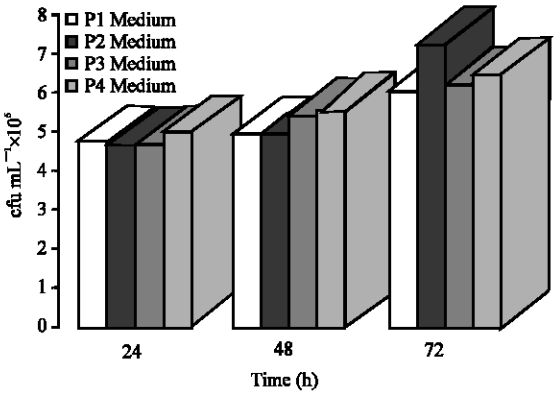

Fig. 3: Viability of the *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains on the P1, P2, P3, P4 media

From the three graphs it results that the milling of the pollen has an essential role in the quick multiplication of the *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains.

Table 1: Several organoleptic features of the symbiotic product on the 4 culture media

	Product homogeneity				Taste			
Time (h)	P1	P2	P3	P4	P1	P2	P3	P4
0	Homogenous	Homogenous	Heterogeneous	Heterogeneous	Characteristic	Characteristic	Characteristic	Characteristic
24	Homogenous	Homogenous	Heterogeneous	Heterogeneous	Sweet and Sour	Sweet and Sour	Sweet and Sour	Sweet and Sour
48	Homogenous	Homogenous	Heterogeneous	Heterogeneous	Sweet and Sour	Sweet and Sour	Sweet and Sour	Sweet and Sour
72	Homogenous	Homogenous	Heterogeneous	Heterogeneous	Sweet and Sour	Sweet and Sour	Sweet and Sour	Sweet and Sour

Table 2: Average values of cholesterol found in the lots of female and male rats treated with symbiotic product

Intake (mg kg^{-1})	Average cholesterol in female rats (mg %)	Average cholesterol in male rats (mg %)
200	30.0	37
20	28.3	27
2	30.7	32
Reference	33.0	33

Other features monitored in obtaining the symbiotic product are homogeneity and taste. The results are shown in Table 1.

Table 1 shows that homogeneity is kept during the entire fermentation period for the P1 and P2 media. On the other two media, homogeneity is not kept.

The taste at the inoculation time is sweet, due to honey and pollen. Once the lactic acid is formed, the taste becomes an appropriate sweet and sour mixture. Besides these characteristics, the aroma of the product was also monitored, which, after 72 h of fermentation, is characteristic to pollen and lactic acid.

Testing of the nutritive value of the symbiotic product: The evolution of the body weight of the rats treated with symbiotic product, obtained on the P1 medium, in intakes of 2, 20 and 200 mg kg^{-1}, respectively, is shown in Fig. 4.

Figure 4 shows that, in all the lots treated with symbiotic product, the weight gain was higher than that of control. This increase of the weight gain is noticed both in females and in males. The average gain of the female lots was of 105% and the average gain of the male lots was of only 103%. For females, the maximum average gain was reached during the third week, being 107%. For males, the maximum average gain was reached during the fourth week, being 105%. The maximum gain was obtained after the 20 mg kg^{-1} intake. It can be noticed that the weight gain was maximum during the third and fourth week of treatment.

Another monitored parameter was the cholesterol level. The determined experimental data is shown in Table 2.

The lowest cholesterol level is found in the lots treated with 20 mg of symbiotic product. Thus, we can conclude that the controlled used has a positive influence on the decrease of the blood cholesterol level. This way, using this product determines a balancing of the intestinal microflora and can determine the decrease of the cholesterol level.

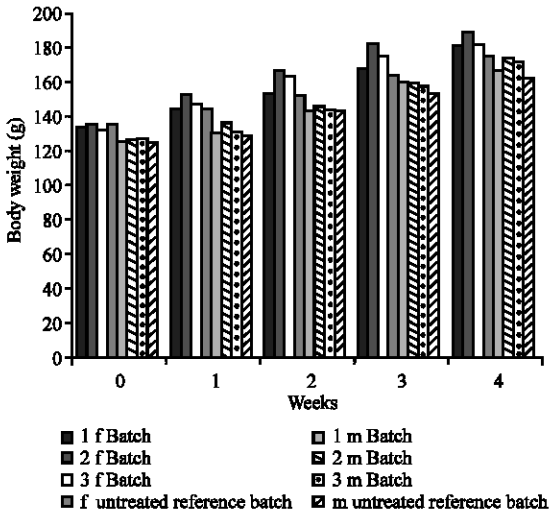

Fig. 4: Body weight of the rats treated with symbiotic product
f-females
m-males
Lot 1-treated with 2 mg of product
Lot 2-treated with 20 mg of product
Lot 3-treated with 200 mg of product

DISCUSSION

The highest viability of the 2 *Lactobacillus* strains is seen when using the 2 culture environments with ground pollen and honey 3% (P2 and P4), as they contain the highest quantity of glucides. Many glucides from pollen and honey are oligosaccharides that can be considered prebiotic, positively influencing the development, the activity and the viability of *Lactobacillus plantarum* 2s and *Lactobacillus acidophilus* 1a strains. And so, the most important result is that the high number of micro-organisms determined by the composition of P2 environment can lower the cholesterol level. So, the composition of the culture environment has an indirect positive effect on the level of cholesterol. If the direct level is exercised on the growth of the milk bacillus number, the indirect one is important as well, as it influences the number of lactic micro-organisms.

In the specialty literature, studies are published regarding the growth and the development of different

lactic bacteria strains in milk products, which contain honey in different percentages. Most of the studies are limited to supplementing a culture environment with a certain quantity of honey. But they are not studies on the growth and development of milk bacillus on pollen and honey. The results obtained on this type of combination of the 2 *Lactobacillus* strains are original, sustained by the composition of the culture environments, the new type of product which can be obtained and by the indirect effect. So, cholesterol lowering can be considered as the first attribute resulting from such a bio-technology process.

CONCLUSIONS

The benefic effects of pollen on health are combined with the positive effect exercised by the microbial biomass. Hence, the synergic effect of the components ensures the functionality of the symbiotic product. The taste, aroma and homogeneity of the product are important commercial features.

In the case of male and female rats treated for four weeks with symbiotic product, the cholesterol level generally decreases in the treated lots, compared to the control, which proves the lack of toxicity of the product. Practically, the values are within the physiological limits in all the treated lots.

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