PERIODICO di MINERALOGIA Volume 89, No. 3, 2020


# Probiotic Product Based on Pollen and Honey

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## Abstract

*In this study, we attempted to find an as good as possible medium, based on grounded and ungrounded pollen, with supplementary honey, in order to obtain a probiotic product. Within the study, we followed the consumption of glucides, the accumulation and the viability of lactic acid.*

Keywords: *Lactobacillus acidophilus, Lactobacillus plantarum,* pollen, Lactic acid

## Introduction

The term *probiotic* mainly refers to living microorganisms that survive crossing the intestinal tract and that exercise positive effects on the organism which swallows them. Probiotic microorganisms are marketed as dairy products, powders or capsules. The positive effects are concentrated on the immune system, cholesterol diminution, cancer, diarrheic accidents. Several probiotic microorganisms can be given as an example: *Lactobacillus rhamnosus, L. acidophilus, L. casei, L. plantarum, Bifidobacterium bifidum, Enterococcus faecium*. [1, 2, 3]

The studies proved that the probiotic microorganisms survival in the gut has good values, between 20 – 40%. The main obstacles against their survival in the gut are gastric acid acidity and gallbladder salts. Recent studies proved that the maximum effect is reached when the microbial cells adhere to the cells of the intestinal mucous membrane**.** Unfortunately, if the cells don't adhere at the gut level, then the positive effect disappears when they leave the gut. This effect is maintained only by continuous ingeration of probiotic microorganisms. This fact can be considered as the „deficiency" of the probiotic microorganisms which don't adhere at the gut level. [4, 5, 6]

The endogen flora and the immune system have an important role in the process of carcinogenesis. They are both influenced by probiotics, so we can conclude that probiotics help in preventing tumors. Many studies proved that probiotics can stop the cancroid process by inhibiting certain substances and enzymes with tumoral effect. However, unfortunately, the studies are not complete and a clear effect of probiotic microorganisms on the cancer cannot be presented. [7, 8, 9]

One of the positive effects very well known is exercised against diarrhoea caused by the use of antibiotics. This inconvenient is due to pathogen bacteria that multiplies in the gut, but recently studies have proved that at a long administration of antibiotics, the bacteria get more resisting. Probiotic microorganisms can inhibit the multiplication of pathogen bacteria by the release of certain inhibiting substances. The positive effects were proved in the case of infections with *Clostridium difficile*, which caused diarrhoea and colic; the negative effects were eliminated by lactobacili administration. Other studies have demonstrated that the

administration of antibiotics, which caused diarrhoea is eliminated by *L. acidophilus* administration. Another type of diarrhoea, traveller's diarrhoea, which emerges with the population of industrial areas and with those who travel to tropical regions, can be treated with lactobacili. This is an alternative to the treatment with medicines. [2, 10, 11, 12, 13, 14, 15, 16]

Thus, these microorganisms are an obstacle against pathogen microorganisms from the gut or in transit through the digestive tube**,** stopping their colonization by occupying their nutritive substrate. Probiotic microorganisms stabilize the pH of the digestive tube, stopping the development of the pathogens and encouraging the development of useful microorganisms. The decrease in the number of pathogen germs, as a result of probiotic administration in food is realized in two ways: 1. Production of substances with antibacterial selective effect (organic acids or hydrogen peroxide; 2. Competition for the situs of fixation on the digestive tube, that is usually won by the more numerous microorganisms. [17, 18, 19, 20, 21, 22, 23]

## Materials and Methods

### 1. The microorganisms

During this study we used two lactic bacteria strains existing in Biotehnol collection: a *Lactobacillus plantarum* 2.2 strain and a *Lactobacillus acidophilus* 1a strain.

### 2. Culture media

The two lactic bacteria cultures are kept on a medium with the following composition: (g/l) 90 g milk powder, 2g yeast extract. The culture keeping takes place at $4^0$C. In order to inoculate the media with pollen and honey we used a fresh developed culture on this medium (48 hours). In order to determinate the probiotic product viability (CFU/ml), we used MRS with the following composition: 1% peptone, 0,5% meat extract, 0,5% yeast extract, 2% glucose, 0,2% $K_2HPO_4$, 0,2% ammonium citrate, 0,5% sodium acetate, 0,1 $MgSO_4$, 0,05% $MnSO_4$, 0,1 ml% Tween 80, pH 6,5. For the solid MRS medium, we added 2% agar-agar and for the preparation of semisolid media we added 0,7% agar-agar.

In order to obtain the probiotic product, we tested for types of culture media that contain ungrounded or grounded pollen and honey that we noted: $P_1$ - 20g ungrounded pollen, 3g honey, 5 ml distilled water, 0,04 g inoculum; $P_2$ – 20g grounded pollen, 3g honey, 5 ml distilled water, 0,04 g inoculum; $P_3$ – 20% ungrounded pollen, 3% honey, 0,04 g inoculum; $P_4$ – 20% grounded pollen, 3% honey, 0,04 g inoculum.

We observed that the media which contain grounded pollen have a greater consistencethan those with ungrounded pollen, though, after approximately 15 – 30 minutes after the obtaining of the medium, the pollen granules hydration leads to their breaking. This phenomenon appears with any of the media, whatever the amount of water in the medium, but the time can be improved if, after adding the pollen granules, the medium is firmly stirred.

The essential variables are the available carbohydrates type and sum, the reserve of the essential aminoacids and the composition of lipids. From this point of view, the obtained media offer all the essential elements for the development of lactobacili strains.

| | | | |
|---|---|---|---|
| $P_1$: | aminoacids and proteins ~ 2,7 g; | sugars ~ 4,9 g; | lipids ~ 0,6 g |
| $P_2$: | aminoacids and proteins ~ 2,7 g; | sugars ~ 4,9 g; | lipids ~ 0,6 g |
| $P_3$: | aminoacids and proteins ~ 1,2 g; | sugars ~ 2 g; | lipids ~ 0,2 g |
| $P_4$: | aminoacids and proteins ~ 1,2 g; | sugars ~ 2 g; | lipids ~ 0,2 g |

**3. The determination of glucides quantity**

The glucides in the medium were determined by the o – toluidine method where: the glucides react with the o – toluidine in a medium of acetic acid, forming a green – blue complex whose intensity is proportional with the reducing the concentration of the glucide. The measurement of the sample's extinction is made at λ = 635 nm. There are two possibilities: one with a preliminary deproteinisation and one without deproteinisation**.** In this study the alternative without deproteinisation was applied.

| | **P (ml)** | **S (ml)** | **B (ml)** |
|---|---|---|---|
| **Sample** | 0,1 | - | - |
| **Glucide standard 100mg%** | - | 0,1 | - |
| **o - toluidine** | 3 | 3 | 3,1 |

The test tubes are kept in a bath of hot water boiling for 8 minutes. They are cooled and then the $E_P$ sample's extinctions and the $E_S$ standard to the blanc at 635 nm in a 1 cm cuve are measured. The $C_P$ reducing glucide concentration expressed in mg reducing glucide/100 ml medium is calculated after the next formula: $C_P = \frac{100 \times E_P}{E_S}$.

**4. The determination of lactic acid quantity**

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

**5. The viability determination**

The determining of the CFU/ml was achieved by successive dilutions methods and then by MRS sowing. For each period the viability determination was achieved three times. The calculation formula was: CFU/ml = $\frac{n}{D} \times 10$, where n – the colonies number, D – the dilution, 10 – a constant for the reportation to 1 ml.

## Results and Discussions

The media employed to obtain the probiotic product had as main component the pollen (poliflora or mountain flowers). The studies focused on finding the best composition of the medium based on pollen and honey, given the nutritive effect of the honey. After identifying the two microorganisms, we found the best temperature of development, $37^0$C; this was the testing temperature. Another important factor was the keeping of the anaerobiosis, given the fact that these two microorganisms develop the fastest in oxygen absence and have a relatively diminished tolerance to oxygen.

The pollen used to obtain the media was employed as such or was ground in a grinder. The hydration of the media is a very important factor in the obtaining of the best culture media based on pollen used in the growing of *L. plantarum* 2.2 and *L. acidophilus* 1a strains because it leads to the unbinding of the pollen granule and to the release in the medium of the useful substances; when the pollen is unground, another important factor is the unbinding of the pollen's granule by the hydrolitic action of microorganisms. These problems are not so important when the pollen used in the composition of the culture media is ground. It is the obtaining of a medium composition with a minimum quantity of water which is aimed at. The role of a minimum quantity of water consists in the obtaining of a product with as great as possible a firmness that is a high concentration not only of the microorganisms number but also a high concentration of the nutritive substances.

The studies were made simultaneously on media based on pollen ground and unground. As inoculum we used a culture of *L. plantarum* 2.2, *L. acidophilus* 1a and a mixture of the two microorganisms. The medium of maintenance of these two microorganisms was used as medium for inoculum: milk powder 9%, yeast extract 0,2%. This form of the inoculum is preferred because the using of products or components as close as possible to the traditional ones is aimed at.

Therefore, for the first time, determinations were made on a substantial medium, hydrated only as much as it is necessary to dissolve honey and hydrate the added quantity of pollen, $P_1$ medium. Using this formula, a mixture with a high firmness is obtained.

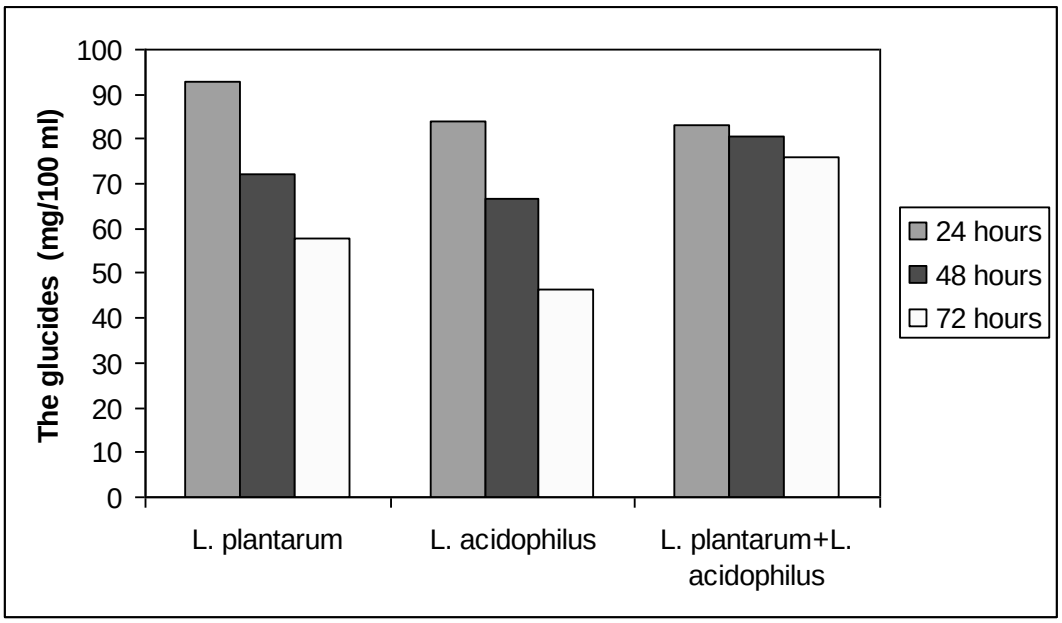


**Figure 1.** The **g**lucides consumption on $P_1$ medium

From **Figure 1** we can conclude that the most diminished consumption took place in the case when the medium was sown with *L. plantarum* and *L. acidophilus* after 72 hours. The best consumption was registered at *L. acidophilus* strain at all lapses of time. Even if for the *L. plantarum* strain the consumption of reducting glucides was more diminished, for the first 24 hours the consumption was faster then in the next 48 hours, which indicates a rapid multiplication of the microorganism during this period. For *L. acidophilus*, the multiplication and the glucose consumption was approximately synchronized. When the medium is sown with the two microorganism, the diminished consumption of reducing glucides can be attributed to the competition between the two microorganisms. The lactic acid accumulation is presented in **Figure 2**.

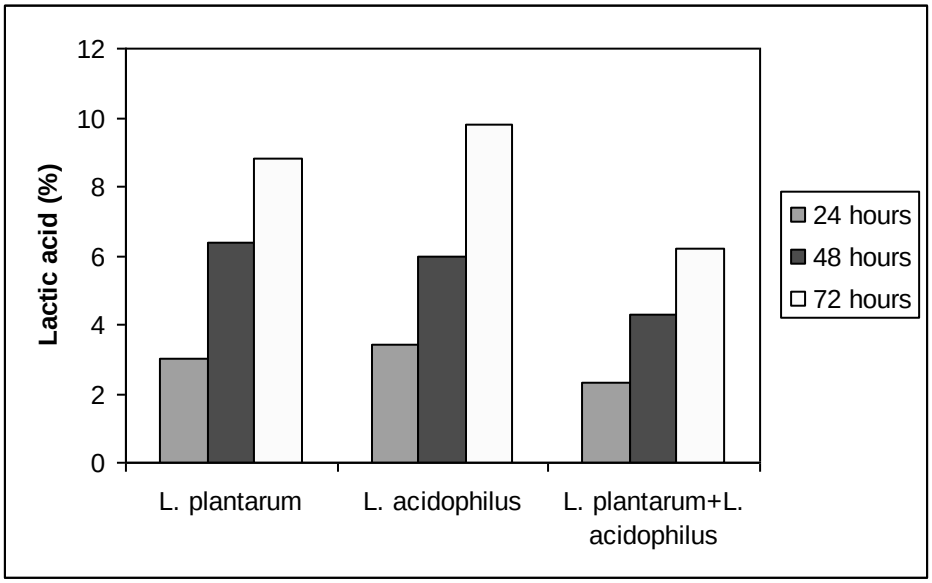


**Figure 2.** Lactic acid accumulation on $P_1$ medium

The lactic acid accumulation is similar to that of **Figure 1**, because the proportions of glucose consumption are kept. The *L. plantarum* strain has a more diminished, but satisfying lactic acid accumulation, in comparison with *L. acidophilus* strain. The *L. plantarum* recorded a greater accumulation at 48 hours of fermentation, but at 24 hours and especially at 72 hours the accumulation of lactic acid is surpassed by the *L. acidophilus* strain. For the medium sown with both strains of acido-lactic bacteria, the results are not satisfactory, even if the accumulation takes place at a relatively constant speed. There are two assumptions for the weaker results: one, the medium which contains unground pollen, though shortly after obtaining of the medium the hydrated pollen granules can break; the second hypothesis can be the competition between the two microorganisms.

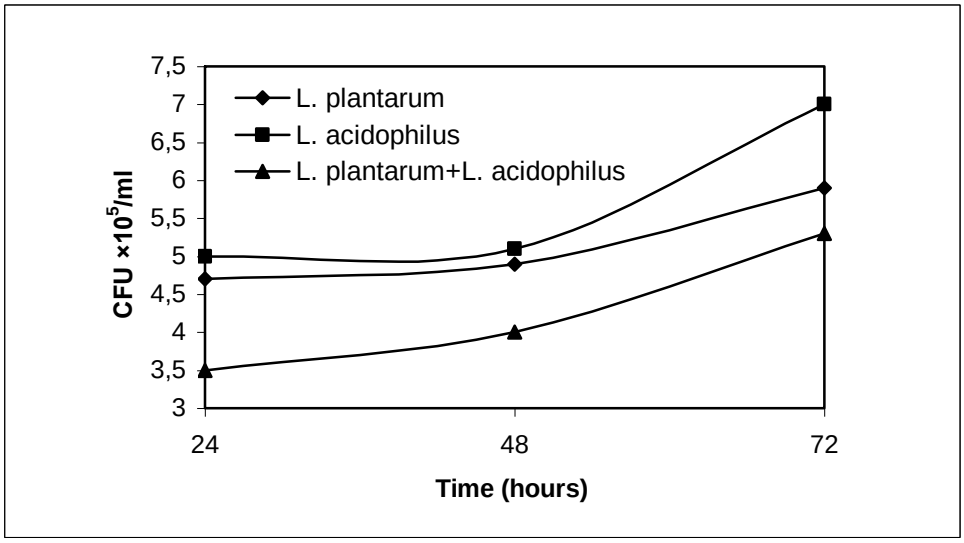


**Figure 3.** The strains viability in case of employing the $P_1$ medium

The viability of the microorganisms on the $P_1$ medium is presented in **Figure 4**. The weakest viability is observed, of course, on the medium inoculated with both microorganisms,

where the competition between them has an important role. Though, in the case of *L. plantarum* strain the viability values are similar to those of *L. acidophilus*, at the end the difference is obvious. The CFU/ml value for *L. acidophilus* strain at 72 hours is $7\times10^5$.

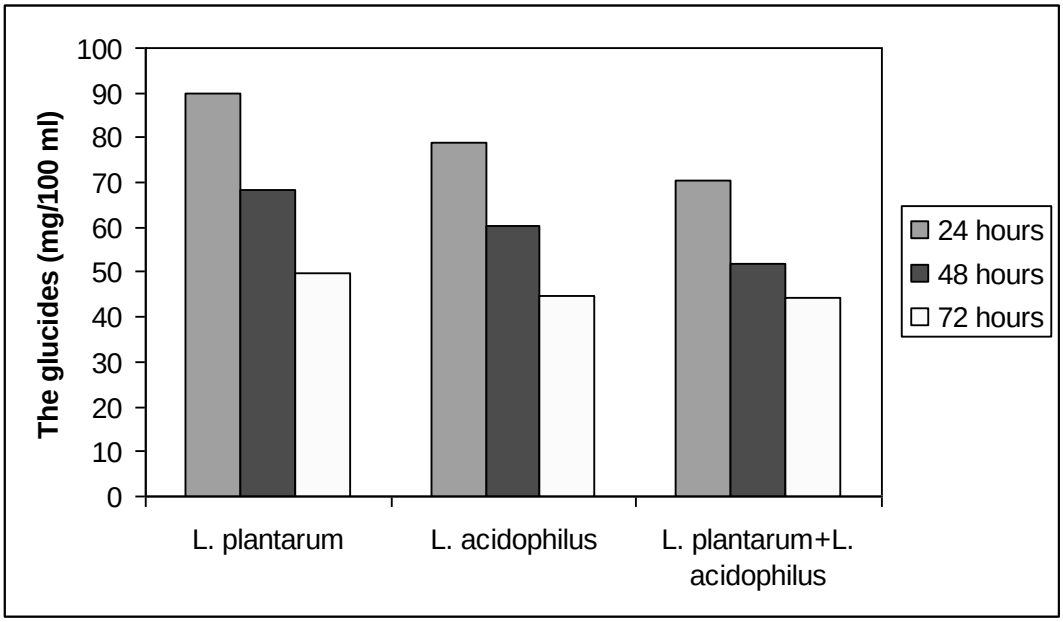


**Figure 4.** The glucides consumption on $P_2$ medium

For the medium that contains ground pollen ($P_2$ medium: 20 g pollen ground, 3 g honey, 5 ml distilled water, 0,04 g inoculum), the results are generally satisfactory, because at 72 hours the consumption of glucides is approximately equal. *L. plantarum* consumed the smallest quantity of reducing glucides, and the greatest consumption was registered at the medium sown with both microorganisms, though the sowing with *L. acidophilus*, as only microorganism, led to a greater consumption. **Figure 4** demonstrates that the medium which contains ground pollen has a positive influence on the multiplication of lactic bacteria, though the same quantity of ground pollen contributes to the growth of medium's firmness. The accumulation of lactic acid in this medium is presented in **Figure 5**.

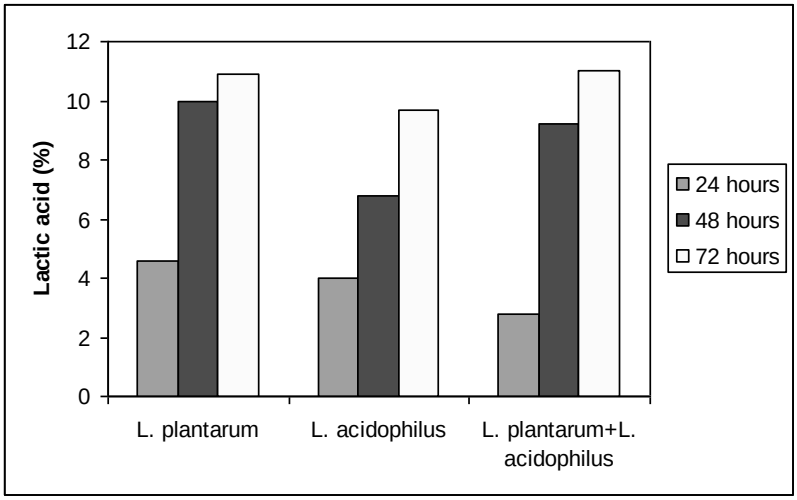


**Figure 5.** The accumulation of lactic acid on the $P_2$ medium

In **Figure 5**, we can notice that the greatest accumulation of lactic acid is given by the sowing alternative with both microbial strains, though at 24 hours the accumulation is the

lowest, but at 48 hours this accumulation surpasses the *L. acidophilus* strain, which is similar with the *L. plantarum* strain. The results obtained on the medium sown with the *L. acidophilus* strain are similar to the *L. plantarum* strain. The results obtained on the medium sown with the *L. acidophilus* strain are weaker, though at 24 hours the accumulation was high enough, but it seems that finally the microorganism adapted harder or it used more reducing glucides for its own development. So, we can consider that the accumulation of lactic acid on the medium sown with two microorganisms was supported more by *L. plantarum*. Though both strains developed, the proportion of the accumulation of lactic acid can not be the sum of the two media, sown with one strain. Yet, it seems that the firmness of the medium and the competition between microorganisms do not have a positive influence on fermentation.

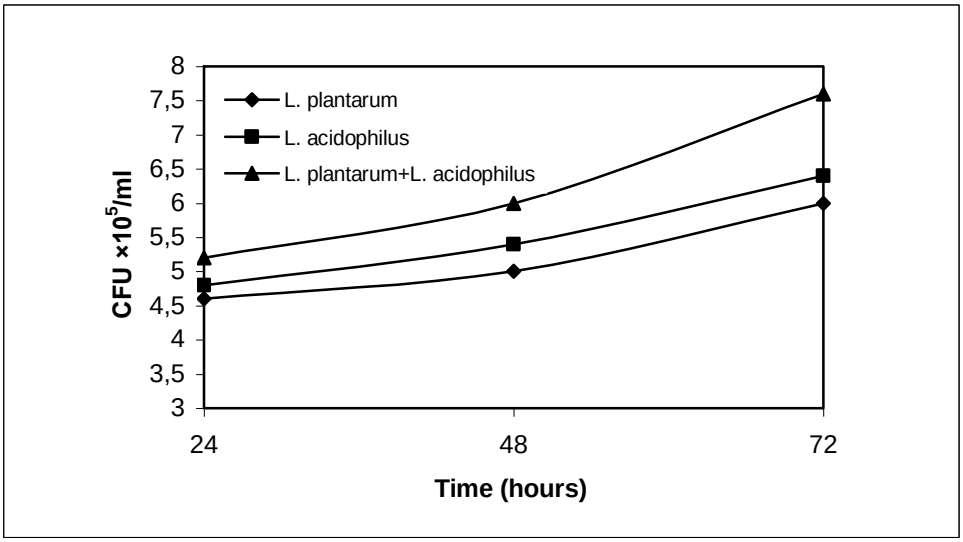


**Figure 6.** The strains viability in case of employing a $P_2$ medium

The microorganisms viability on $P_2$ medium, **Figure 6**, is influenced obviously by the medium composition. We can observe in the figure that, for the *L. plantarum* strain, the pollen grinding in the medium is not an important factor, because the strain maintains relatively constant viability values. The pollen grinding has an important role in the case that the $P_2$ medium is inoculated with both microorganisms, the viability having values of $7{,}6\times10^5$ at 72 hours. For *L. acidophilus* strain the viability values, relatively decreased comparatively to the cultivation on the $P_1$medium are constant, but insignificant.

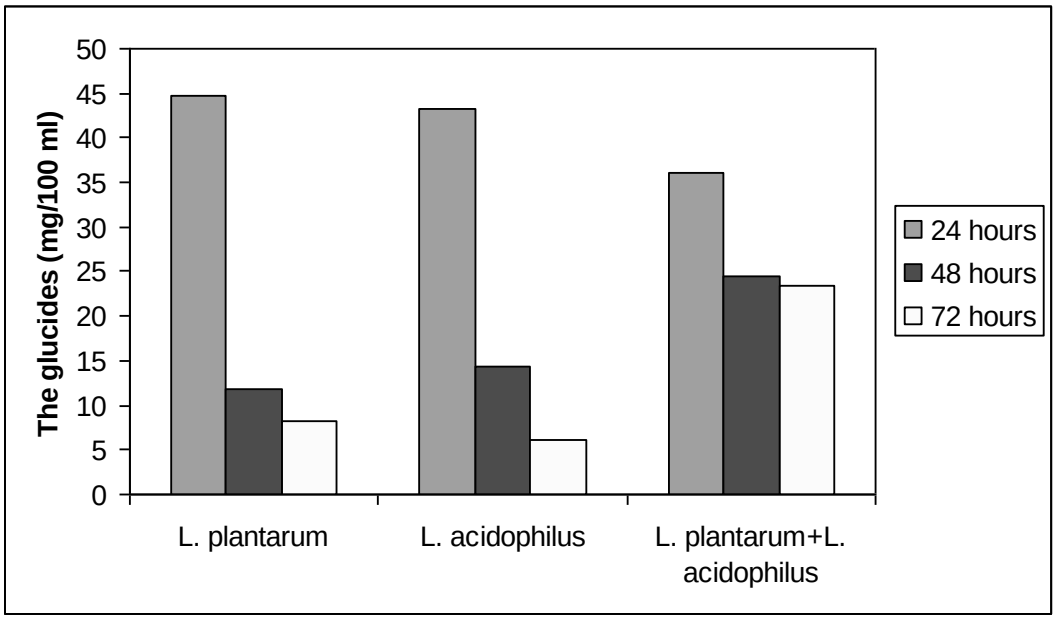


**Figure 7.** The consumption of glucides on a $P_3$ medium

From **Figure 7**, based on $P_3$ medium, we can observe a high consumption of reducing glucides between 24 – 48 hours at the media inoculated with *L. plantarum* and *L. acidophilus*. The highest consumption is observed with the medium inoculated with *L. acidophilus*, but the results are approximately equal with the medium inoculated with *L. plantarum*. The conclusion can be that a high hydration of the medium, similar with that of the classical media, leads to a very good development of the two microorganisms. In the case of inoculation with both microorganisms, the consumption is better then for the first medium's alternative, but the difference is anyway unpropitious, in comparison with the medium sown with one microorganism. The quantity of water, higher than that of the initial formula, ensures a higher speed of the fermentative process and it also allows the stir of the medium within the limits of reasonable power consumption. Although, to approach to the physical properties of the pasture harvested from the honeycomb, further processing that will eliminate the water in excess is necessary. The accumulation of lactic acid is presented in **Figure 8**.

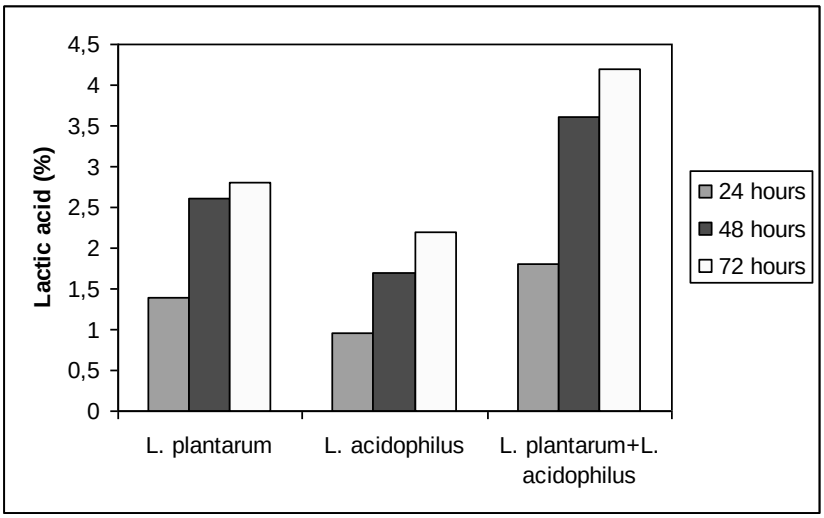


**Figure 8.** The accumulation of lactic acid on a $P_3$ medium

The results presented in **Figure 8** are opposite to those presented so far, which means that the inoculation with both microorganisms leads to the obtaining of a quantity of lactic acid that can be considered the result of the sum of the accumulation of the two strains. Though the consumption of reducing glucides was not considerable, the resulted quantity of lactic acid is important. A possible conclusion is that a big quantity of glucides was used for the multiplication and the development, for inoculations with one microorganism and not for the synthesis of the lactic acid. For this medium, the strain that produces a higher quantity of lactic acid is *L. plantarum*. An important contribution in the production of the lactic acid is the type of medium, because they are not classical media and the strains must adapt to the new nutritional sources.

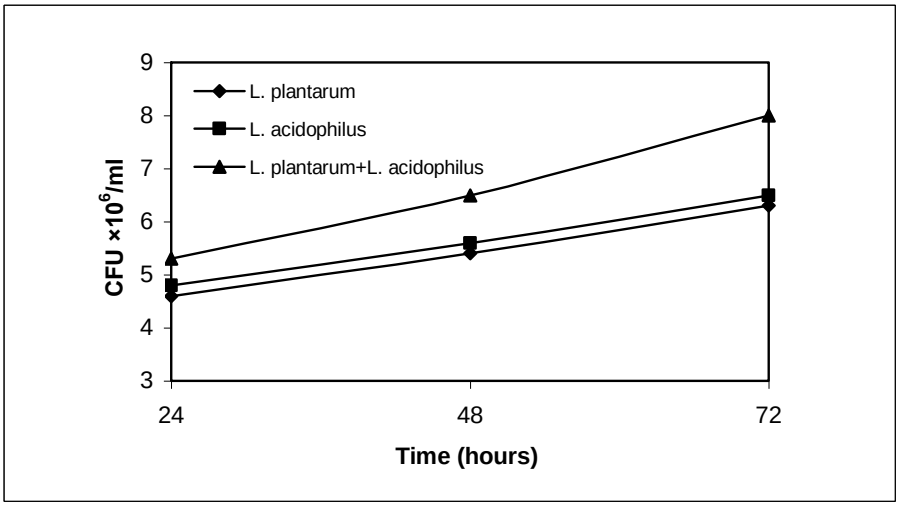


**Figure 9.** The strains viability in case of employing a $P_3$ medium

The viability values on a $P_3$ medium, **Figure 9**, are the clear result of a medium that is close to normal one. The bigger water quantity influences positively the viability, this one being better when both microorganism are used for inoculation. When a single microorganism is used, the viability is approximately constant. Both strains have big CFU values, but none compares with that obtained for the inoculation with both microorganisms, $8\times10^6$.

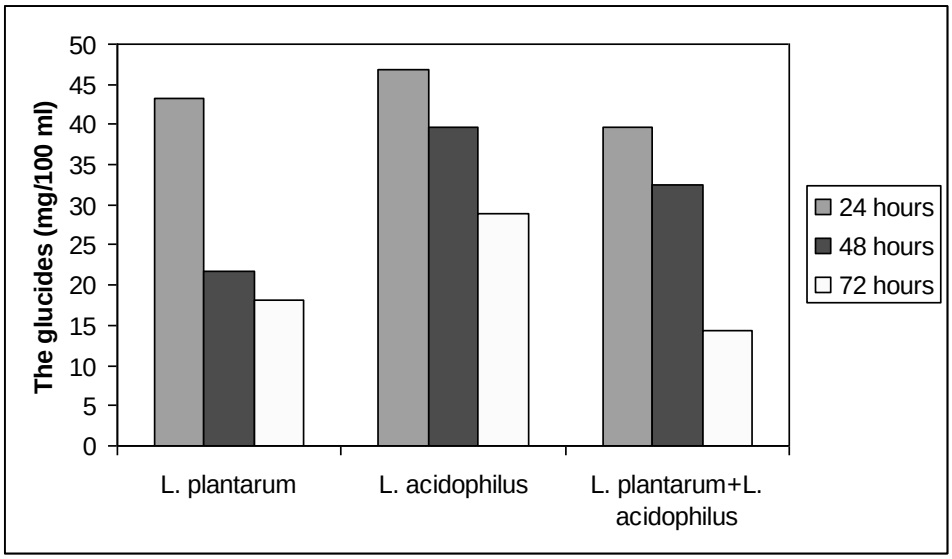


**Figure 10.** The glucides consumption on a $P_4$ medium

From **Figure 10,** based on a $P_4$ medium, one can see that the milling grinding of the pollen, before its introduction in the medium, leads to a similar development of the strains concerning the glucose consumption, though the *L. acidophilus* strain has a low consumption. The most important diminution is noticed with the medium inoculated with both microorganisms. From the figure it results that the strain with a significant multiplication up to 48 hours is the *L. plantarum* strain.

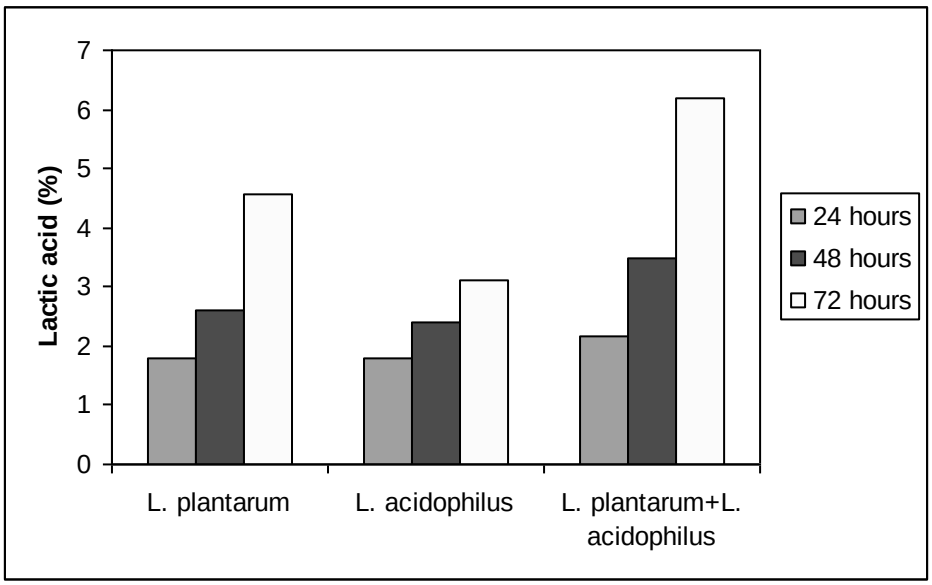


**Figure 11.** The accumulation of lactic acid on a $P_4$ medium

This figure is in correlation with **Figure 10** because the highest accumulation of lactic acid was achieved on the medium inoculated with both microorganisms. The accumulation is approximately equal with the sum of accumulations obtained on the media inoculated with one microorganism. The highest accumulation is registered after 48 hours that is after a significant multiplication of the microorganism. Another important conclusion is the significant contribution of *L. plantarum* strain at the final quantity of lactic acid, but the role of *L. acidophilus* strain must not be neglected, because, if it doesn't produce a significant quantity of lactic acid, it is important thanks to the positive influence on the human organism.

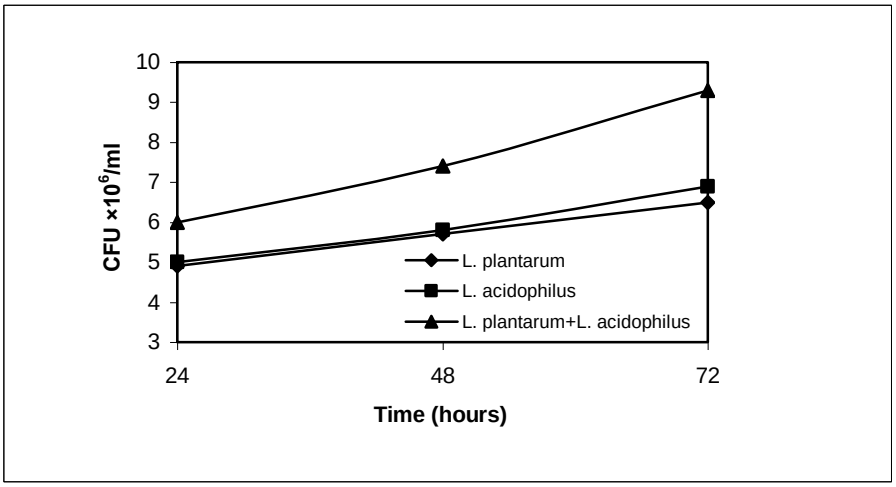


**Figure 12.** The strains viability in case of employing a $P_4$ medium

The viability results on $P_4$ medium, **Figure 12**, are a little better than those obtain on $P_3$ medium and they are influenced by the pollen grinding. These grinding determines a better utilization of the food sources and thus the multiplication of the microorganisms is influenced positively. For the inoculation with a single microorganism, the best results are obtained in the case of *L. acidophilus* strain while the *L. plantarum* seems more preoccupied by the lactic acid biosynthesis. The values are constant and a little higher than in case of employing ungrounded pollen. At 72 hours, the viability in the case of inoculation with both microorganisms is $9{,}3\times10^6$, thus the utilization of both strains proves most propitious for this medium type.

From a technological point of view, better results were obtained by using media that contain a higher quantity of water, these media being close, by their physical properties, to the media traditionally used in biotechnologies.

The physical properties (humidity, milling/grinding degree) of the raw material, in this case the pollen, have an important influence in the obtaining of a higher quantity of lactic acid and, this way, a more efficient using of reducing glucides. This fact can be observed if we estimate the necessary times for obtaining of best quantity of lactic acid, comparing the evolution of the process between the data obtained on $P_1$ and $P_3$ media, on the one hand to that of the $P_2$ and $P_4$ media on the other hand.

The active microorganisms interact intensely with the environment by the exchange among the elements of the medium, having the purpose of obtaining metabolic products. The chemical composition of the products is very important for the metabolic activities of the microorganisms.

When the probiotic bacteria participate actively at the fermentation, the aspects of the food composition and interactions between foods must be considered at a high intensity. The antagonism between the probiotics and the starter cultures will have as effect the growth's slowness or the complete inhibition of one bacterial element. An important aspect is the producing of lactic acid and the considerable pH reduction during fermentation that has as result the inhibition of probiotic organism.

The physiological condition of the probiotics is very important when the fermentation is considered finished. Many investigations demonstrated that the bacteria in logarithmic phase are more sensitive to the stress than the bacteria in stationary phase.

In the experiments with starter organisms we observe that the environment's factors and the bacteria's signals at the passing from the logarithmic to the stationary phase can have an important effect on the survival rate during the stationary phase. The researches concerning the regulation of the stationary phase at lactic bacteria represent a new discipline. Many researches are necessary in order to exploit the possibilities of improvement of the survival rate not only to the probiotic bacteria, but also to the traditional starter bacteria.

## Conclusions

The pollen's advantages for health are combined with the positive effect exercised by the microbial biomass. The most propitious alternative for the media with a higher concentration of pollen ($P_3$ and $P_4$) is *L. acidophilus*, and for the lower concentrations of pollen the most propitious is the alternative of using an inoculum that contains *L. acidophilus* and *L. plantarum*. For the determination of this best alternative, there were considered the elements of commercial availability of the microbial strains. The biggest viability values on the media containing more water are an important factor, but the final decision a must consider much more factors. We can conclude that the media with less water are the most propitious for the product obtained, the microorganisms' viability having best values, though

these values are not very high. The pollen grinding on $P_2$ and $P_4$ medium seems to be an essential factor in the obtaining of the expected results.

In order to estimate the combined effect of the microbial strains, with positive results (the accumulation of lactic acid on $P_2$, the glucose consumption on $P_3$, the accumulation of lactic acid on $P_3$ and $P_4$) we can consider that this effect is similar with the effect proceeded naturally in the honeycombs in the beehives.

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