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STUDIES CONCERNING THE CHARACTERISATION OF A PROBIOTIC PREPARATION, CONTAINING A MIXTURE OF *LACTOBACILLUS* PURE BIOMASS AND MEDIA WITH POLLEN AND HONEY

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Abstract

This paper presents the results obtained on the last years in the frame of a National Research Project which has in view the biotechnological studies concerning a probiotic manufacture on media with pollen and honey, using some selected bacterial strains and several essential factors for direct and effective microbial biomass production. A set of chemical and pharmacological analyses was established, in order to substantiate scientifically the valuable content of this type of probiotic preparation, which can be used in order to improve the balance of intestinal microflora. Our final results were subsequently used for an invention demand concerning a lactobacilli biomass having a strictly determined microbiological and chemical composition and used as the main component of a probiotic preparation, which was approved by our National Authorities and which has obtained the Silver Medal and the International Award of the Industrial Institute of Agricultural Engineering Poznan - Poland - "Medal Product" at the International Contest Innova-Eureka, Brussels, 2011.

Key words*: probiotic, characterisation, lactobacillus, pollen, honey.*

INTRODUCTION

The technology concerns to a lactobacilli preparation, having a strictly determined microbiological and chemical composition, used as component of a probiotic obtained on media with pollen and honey.

The study describes several essential factors for direct and effective microbial biomass production using some selected *Lactobacillus* strains, while the obtaining of a pure biomass preparation (conditioned in a solid shape) involves 3 steps of centrifugation using for each of them increasing speeds. The copper, zinc, cobalt, cadmium and iron total concentrations were determined by A Perkin Elmer Elan DRC-e inductively coupled plasma spectrometer ICP-MS and by FAAS.

The obtained Lactobacilli preparation was subsequently used for a complex probiotic manufacture on media containing pollen and honey. Probiotics are containing live non-pathogenic microorganisms that benefit by improving the balance of intestinal microflora. They may either be bacteria: often lactic acid producing such as *Lactobacilli* (including acidophilus), Bifidobacteria, and Streptococci, or yeasts such as *Saccharomyces boulardii*. These organisms must have the following properties: be of human origin, resist acid and bile, and adhere, colonize and survive in the human intestine, despite the increased level of honey's content (Haller et al., 2010, Hedin et al., 2007).

Toxicity testing of a compound entry is required and previous an appearance pharmacodynamic study which shall be demonstrated. At this stage has been tested under toxicological aspects the sample-test BIOSIN, having the following composition: 20 g ground pollen, 5 g honey, 5 ml of distilled water, 0.1% inullin. This sample was inoculated in the ratio of 1% by the following mixture: 10% *Lactococcus fermentum BS2*, 15% *Lactobacillus BS1*, 15% Lactobacillus BS3, 10% *Lactobacillus paracasei BS6*, 25% *Bifidobacterium bifidum BS4*, 25% *Bifidobacterium bifidum BS5*. Test sample shall

be presented in liquid form, viscous grease has (paste) brown, water-dilutable varnish primer.

MATERIALS AND METHODS

Lactobacillus liquid conserved culture were pipetted aseptically into the specific culture medium (500 ml flask containing 100 ml liquid medium) in order to prepare an inoculum. For the preparation of the bacterial biomass, each 20 ml of *Lactobacillus* or *Bifidobacterium* starter liquid culture were transferred aseptically into the culture media (750 ml flask containing 200 ml liquid medium). All the media were previously sterilized 20 min at 110°C. After 48 hours of static cultivation and the conversion of the liquid cultivated media to a solid pure preparation (which means without any trace of the cultivation media), the copper, zinc, cobalt, cadmium and iron total concentrations were determined by A Perkin Elmer Elan DRC-e inductively coupled plasma spectrometer ICP-MS and by FAAS.

The product with a probiotic action taken in this study (sample 1), consists of a lyophilized biomass of lactobacilli and *Bifidobacterium*, pollen, honey and inullin. An assessment of trace elements and macroelements represents a priority at the international level, because there is a very narrow demarcation between the toxic compounds and the essential elements.

In the literature, they mention that the techniques of atomic spectrometry are the most widely used for the analysis of elements at various levels of concentration. For the pharmaceutical products and medicines, a main importance is represented by flame atomic absorption spectrometry (FAAS) or in the oven of graphite (GF-AAS), atomic emission spectrometry in coupled plasma (ICP-AES) and mass spectrometry in inductive coupled plasma (ICP-MS)

Generally speaking, FAAS technique is used in the case of determining concentrations of analyte sufficiently large, and the technique GF-AAS for the low ones, in different matrixes.

The experimental model of our specification from the point of view of the elements, is based on the drafting of two methods, by two advanced analytical techniques:

- mass spectrometry in inductive coupled plasma (ICP-MS) for the evaluation of lead, cadmium, nickel, chromium;
- flame atomic absorption spectrometry (FAAS), in flame of nitrous oxide (acetylene, respectively air-acetylene flame) for the determination of calcium and respectively magnesium.

In order to develop methods for the determination by ICP-MS and FAAS, our studies have focussed on two directions:

a) to identify specific interferences by technique ICP-MS and FAAS;

(b) the development of an analytical protocol for the determination by ICP- MS and FAAS, based on the selection and the establishment for:

- specific conditions for the processing of samples;
- optimal conditions of the operational parameters;
- conditions of quantification (the suitable isotope and the method of measurement of the concentration).

Acute toxicity is targeting toxic effects which appear for a period of 14 days of the administration of a single dose of a product test.

In the case when the substance (product) induce lethality, the lethal dose 50 (LD50) can be established.

It has pursued the highlight of the toxic aspects consecutive a single dose administration of the product test, on laboratory animals, orally.

For this study there have been used mice Swiss, young people, of either sex, with weights 18-20 g, and Wistar rats, young people, of either sex, with weights of 80-100 g, provided from Cantacuzino Institute. The animals have been accommodated in appropriate cages, broken down into groups of the same sex. Experimental room temperature was 22± 2 °C with a relative humidity of 50-60%. The lighting has been achieved artificially, alternating 12 hours light with 12 hours dark after. Standardized animals has received food and water ad libitum. With 12 hours earlier test, they have been kept in post, and is only allowed access to water. The test has been administered by the oral route, similar to methods of administration in humans. For management, the product-test has been dissolved in distilled water as it represented an

optimal vehicle.
The doses administered were 7000 mg/kgc mouse and 5000 mg/kgc in the rat. After administration, the animals have been monitored during the period of the first 8 hours on each hour, and then for the subsequent period of 14 days, at least two times daily. In both mice and rats there were not noted signs of toxicity, changes of behavior spontaneously or food habits and neither lethality.

RESULTS AND DISCUSSIONS

Table 1. The Cu, Zn, Co, Cd and Fe determination

Element	Sample 1 *Lactobacillus plantarum* (ppm)	Sample 2 *Bifidobacterium* (ppm)	Sample 3 *Lactobacillus paracasei* (ppm)
Cu	17.62	17.02	45.54
Zn	38.59	37.98	62.18
Co	5.05	5.02	Under 1.0
Cd	0.06	0.05	0.30
Fe	668.96	664.24	183.17

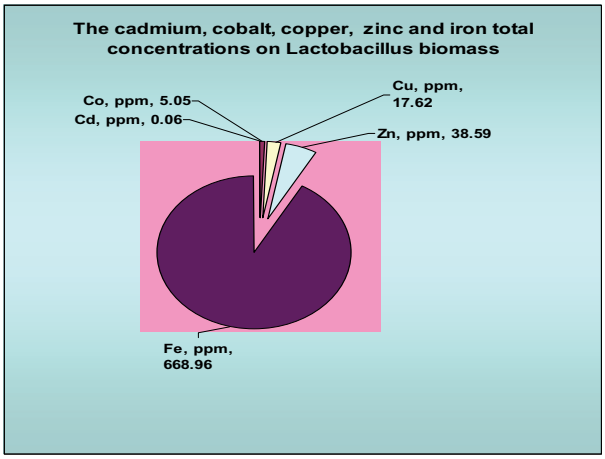

Figure 1. The Cu, Zn, Co, Cd and Fe determination from a *Lactobacillus plantarum* strain biomass

There was developed a method of simultaneous determination of trace elements, by mass spectrometry in inductive coupled plasma (ICP-MS), at very low levels, in order to assess the character as a toxic compound (such as the lead, nickel, cadmium) or as a bioelement (chrome). The developed ICP-MS method allows the analysis of toxic trace elements in the 0.02-0.40 ppm domain, and it can demonstrate by this way the safety for human health of our proposed probiotic product.
Moreover we has developed a method for the individual determination of some macroelements, by flame atomic absorption spectrometry (FAAS), in order to highlight the cations playing a role of essential elements, inside the probiotic preparation.
The results obtained from acute toxicity experiment have provided informations which can not lead to determining the LD50, due to the absence of lethality, under the conditions of maximum manageable doses, from the point of view of physical properties of the samples, by oral use on the selected species
It can be said that the LD50 is situated at higher doses than maximum manageable orally in conditions of this experiment.
Our final results were subsequently used for an invention demand concerning a lactobacilli biomass having a strictly determined microbiological and chemical composition and used as the main component of a probiotic preparation, which was approved by our National Authorities and which has obtained the Silver Medal and the International Award of the Industrial Institute of Agricultural Engineering Poznan-Poland-"Medal Product" at the International Contest Innova-Eureka, Brussels, 2011.

Figure 2. Probiotic product

Advantages of this inventions are:
- the best formula of the cultivation media (for lactobacilli biomass manufacture) was simplified to only glucose, peptone and yeast extract.
- the analytical determination indicates the presence of the most valuable trace elements, wich are requested for an efficient human treatment, as a probiotic preparation: Cu, Zn, Co, Cd and Fe (Figure 1, Figure 2).
- the obtained Lactobacilli preparation was used for an improved probiotic manufacture on media containing pollen and honey.

CONCLUSIONS

The best results for fermentation were obtained at temperatures of 37-40 °C for inoculum, 48-50°C durring the fermentation process and by pH buffering with $CaCO_3$.

The preinoculum media contained malt extract, and the inoculum one of: glucose, peptone and yeast extract.

The best formula of the cultivation media was simplified to only glucose, peptone and yeast extract.

The obtaining of a pure biomass preparation (conditioned in a solid shape) involves 3 steps of centrifugation using for each of them increasing speeds.

The obtained Lactobacilli preparation was used for an improved technology used in order to obtain a new probiotic preparation on media containing pollen and honey.

According to the classification Hodge-Sterner, our probiotic sample-test can be framed within the category of products free of toxicity in the experiment's conditions, the maximum doses manageable orally being accompanied by the lack of any lethality.

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