UNIVERSITY OF ECONOMIC SCIENCES AND PUBLIC ADMINISTRATION

Isolation, identification, physiological, biochemical and functional characterization of Bifidobacteria

Theses of the PhD dissertation

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1. Background and aims of the work

In the past few decades there is an ever increasing interest in healthy nutrition. It is proven that improper eating habits may lead to the development of diseases, and on the contrary: with a healthy diet one is given the chance for a longer and healthier life. The basic principal of a healthy diet is that one should consume the proper amount of each nutrient. There are scientific evidences that not only the energy and nutrient intake have disease preventive effect, but other components of food, as well. Foods that contain components with such beneficial physiological effects, or they are supplemented with them, are called functional foods.

The market of functional foods is continuously growing, now it exceeds 33 billion USD on a world wide scale of which the share of Europe is about 2 billion dollars. Out of the 2 billion USD pre- and probiotic products represent 1.35 billion USD, thus it is safe to say that health of the intestinal tract is a key area within the research field of functional foods.

The microbiota of the human colon is very complex. Among the more than 500 species there are such beneficial bacteria that synthesize different short chain fatty acids during their carbohydrate and protein metabolism, by which the pH of colon decreases and they outplace bacteria that produce toxins and harmful N-nitroso compounds. For this reason it deserves attention that how the change of diet influences the composition of intestinal flora. The goal is to increase the number and the activity of bifidobacteria and lactobacilli in the intestines.

Nowadays, there are many fermented dairy products on the market. Those that contain lactobacilli and bifidobacteria of human origin and posses beneficial characteristics are called probiotic products. The indigestible food components that selectively support the growth of probiotic bacteria are called prebiotics. These are mainly oligosaccharides. Many researcher works on the isolation and investigation of newer and newer probiotic strains and on the production of prebiotics that selectively support them. The most important requirements probiotic micro-organisms must meet are that they have to be of human origin, have to survive the passage through the upper intestinal tract, they should produce short chain fatty acids in order to reduce pH to prevent the growth of pathogens, and they have to adhere well to the intestinal epithelial cells. These imply that putting a probiotic strain into industrial practice requires extremely lot of research and development.

At present the most often used *Bifidobacterium* species is the *B. lactis* in the diary industry. Producers usually choose this strain for its good acid and oxygen tolerance. But, numerous researchers disprove the human origin of this strain. In my work I wanted to isolate and study bifidobacteria of human origin that not only comply with the requirements of probiotics, but possess excellent technofunctional properties.

Main goals of the research work

- Elaboration of methods for the isolation of bifidobacteria from human and food origin
    - Classification of the isolates by molecular genetic method
    - Elaboration of alternative methods for the species level classification of bifidobacteria
- Investigation of the utilization of prebiotic oligosaccharides by bifidobacteria and enteropathogens
- Characterization of the physiological properties of Bifidobacteria
    - Antagonistic effect to enteropathogens
    - Adhesion to human tissue culture
- Investigation of the physiological effect of Bifidobacteria in animal feeding experiments
- Investigation of the survival of Bifidobacteria in experiments simulating the intestinal tract
- Investigation of the effects of Bifidobacteria in the colon in *in vitro* experiments

2. Materials and methods

Bifidobacterium and enteropathogens strains used in the experiments are from different culture collections.

The applied chemicals were purchased from the Sigma, Merck, Reanal, Oxoid and BBL companies.

The anaerobic cultivations were performed in Anaerob Jar + GasPak system (Oxoid) and in anaerobic workstation (Bugbox, Ruskin Technology).

The *Bifidobacterium* strains were isolated on NNLP, GL and DP selective media.

The isolates were identified by Fructose-6-phosphate-phosphoketolase test, by microtiter plate method based on fermentation abilities, by Fourier Transformation Infrared Spectroscopy and by 16S rDNA analysis.

In the prebiotics utilization experiments fructo-oligosaccharides (Raftiline, Raftilose), lactosucroses, isomaltose-oligosaccharides (Oligotime, OligoMT, Isomalto500) and xilo-oligosaccharides were tested.

Antagonistic effect to enteropathogens was investigated by *in vitro* plate method.

Adhesion was tested on HeLa, HT29, Hep2 and CaCo-2P cell lines.

Animal feeding experiments were performed on healthy and on antibiotic treated mice; the groups were fed pro-, pre- and synbiotic food for 4 weeks, and change in the composition of the intestinal microbiota was followed.

Surviving ability in the course of passing through the digestive tract and effect on the colon were investigated on TIM models in the TNO institute in the Netherlands. TIM models are *in vitro* laboratory model systems simulating the digestive tract.

3. Summary of results

Methods for the species level identification were elaborated. One of the methods is based on the examination of fermentation spectrum and breakdown of arginine, the other one is the application of the Fourier Transformation Infrared Spectroscopy (FT-IR). In case of the first method I processed the data available in the literature, and I examined the fermentation abilities of isolated and type strains of bifidobacteria, and created a database. The database contains 54 different characteristics of 65 reference strains. Computer software was written that compared the same 54 characteristics of the unknown isolate with that of the strains included in the database, and it classified the unknown *Bifidobacterium* strain into the appropriate species. In case of the FT-IR spectroscopic method first I determined what medium composition provides the required amount of biomass for the successful execution of the examination. Then I created a spectrum library by taking the spectrum of 99 *Bifidobacterium* strains originating from various places. Validation of the adequacy of the method showed 100% for most of the species except for *B. infantis/longum* it was 80%. Also, in case of *B. pseudocatenulatum* the classification was uncertain due to the heterogeneity of the strains belonging to this species.

I have isolated *Bifidobacterium* strains from human faeces and from food samples and I identified them with molecular biological methods, and with the two above mentioned methods. *Bifidobacterium* strain collection was created containing 32 strains of human origin and 27 strains of food origin. Nine strains out of the 32 isolates proved to be *B. bifidum*, 6 strains were *B. breve*, 4 strains were *B. dentium*, and 11 strains belonged to the *B. infantis/longum* species. Classification of two strains remained uncertain; they were only verified to belong to the *B. angulatum* group. Of the 27 strains of food origin 2 proved to belong to the *B.infantis/longum* species, and 25 to the *B. lactis/animalis* species.

Investigating the oligosaccharide utilizing ability of each strain it was determined that all the prebiotics sold as oligosaccharide were utilized by the bifidobacterium strains as sole carbon source. The oligotime syrup was considered to be the most appropriate one for use in the food industry, because it supported the growth of enteropathogenic strains to a lesser degree than the other investigated oligosaccharides.

In the course of investigating the interaction between bifidobacteria and enteropathogenic strains in some cases certain bifidobacteria exerted antagonistic effect on the enteropathogenic bacteria.

One important criterion of probiotic strains is the adherence to the intestinal cells, because it ensures that they will not be washed out of the body. Determination of the adhering ability of the Bifidobacterium strains was performed on human cell cultures. I found that the adhering ability is a strain dependent characteristic and certain isolates of human origin adhered better to the human cell cultures than the type strains or the strains of food origin.

Animal feeding experiments were performed in which mice were fed with probiotic, prebiotic and synbiotic food, and change of the microbiota of faeces was followed for four weeks. In the first set of experiments healthy mice were tested, while in the second one mice whose microbiota was previously eliminated by antibiotics. Due to the feeding with probiotic, prebiotic and synbiotic foods composition of the intestinal flora of healthy mice did not change, but the healthy balance of the microbiota of antibiotic treated mice was recovered.

I investigated the surviving ability of some isolates in *in vitro* models. The surviving ability is a strain dependent characteristic, as well, and according to my results the *B. bifidum* B7.1 strain is very promising in this aspect.

The probiotic characteristics of the previously selected strains were verified in an *in vitro* model that simulates the functioning of the human colon. In the course of the simulated feeding experiment I examined the composition of the microbiota, and the synthesis of the short chain fatty acids (SCFA), lactate and ammonia. Most important result was experienced in the synthesis of short chain fatty acids. Considering all the produced SCFA (acetate + propionate + butyrate), more of them were synthesized during the investigation of the effect of prebiotics, probiotics and symbiotics than in the control experiments. Short chain fatty acids are synthesized in the anaerobic fermentation of carbohydrates in the colon. Their biological role is extensive: they influence the growth of bacteria that compose the microbiota of the colon; they decrease the possibility of the utilization of alkaline cytotoxic compounds; they serve as nutrient for the cells of the colon wall, the colonocytes, and they reversibly and irreversibly prevent the division of colonocytes.

4. Application of results and the possibility of further development

- It will be possible to identify unknown *Bifidobacterium* isolates of human and food origin with the elaborated classification methods based on fermentation abilities and the Fourier Transformation Infrared Spectroscopic measurement.
- It will be possible to improve the adequacy of identification by completing the database with the FT-IR spectrum of Bifidobacteria of different origin.
- It will be possible to elaborate technologies for the production of dietary supplements, food additives, probiotic or symbiotic foods with the isolated *Bifidobacterium* strains once the necessary authorizations are received.
- By micro-encapsulation it will be possible to protect those strains during the passage in the intestinal tract that do not tolerate it, but possess promising probiotic characteristics, i.e. adherence, antagonistic effect to enteropathogens and increased production of short chain fatty acids.

5. New scientific results

1. Method for the species level identification of bifidobacteria was elaborated that is based on the examination of fermentation spectrum and breakdown of arginine. By using the data available in the literature, and by examining the fermentation abilities of isolated and type strains of bifidobacteria I have created a database. The database contains 54 different characteristics of 65 reference strains. Computer software was written that compared the same 54 characteristics of the unknown isolate with that of the strains included in the database, and it classified the unknown *Bifidobacterium* strain into the appropriate species.
2. Procedure was elaborated for the species level identification of bifidobacteria by Fourier Transformation Infrared Spectroscopy (FT-IR). In this I have determined what medium composition provides the required amount of biomass for the successful execution of the examination. Then I created a spectrum library by taking the spectrum of 99 *Bifidobacterium* strains originating from various places. Validation of the adequacy of the method showed 100% for most of the species except for *B. infantis/longum* it was 80%. Also, in case of *B. pseudocatenulatum* the classification was uncertain due to the heterogeneity of the strains belonging to this species.
3. I have isolated *Bifidobacterium* strains from human faeces and from food samples. The strains were identified with molecular biological methods, and with the two above mentioned methods. *Bifidobacterium* strain collection was created containing 32 strains of human origin and 27 strains of food origin. Nine strains out of the 32 isolates proved to be *B. bifidum*, 6 strains were *B. breve*, 4 strains were *B. dentium*, and 11 strains belonged to the *B. infantis/longum* species. Classification of two strains remained uncertain; they were only verified to belong to the *B. angulatum* group. Of the 27 strains of food origin 2 proved to belong to the *B.infantis/longum* species, and 25 to the *B. lactis/animalis* species.
4. I examined the oligosaccharide utilizing ability of each strain, their antagonistic effect on enteropathogens, their adhering ability on human cell cultures, and their surviving ability in *in vitro* models simulating the digestive tract. The oligotime syrup was considered to be the most appropriate one, because it supported the growth of enteropathogenic strains to a lesser degree than the other investigated oligosaccharides. According to my examinations certain isolates exert antagonistic

effect on the investigated enteropathogenic bacteria. I found that the adhering ability of certain isolates of human origin is better to the human cell cultures than the type strains or the strains of food origin. The surviving ability is a strain dependent characteristic, as well, and according to my results the *B. bifidum* B7.1 strain is very promising in this aspect.

5. Probiotic characteristics of the B7.1, B3.2 and A1.2 strains were proven in *in vitro* models that simulate the functioning of the human colon. In the course of these experiments I examined the composition of the microbiota, and the synthesis of the short chain fatty acids (SCFA), lactate and ammonia. The most favourable result was experienced in the synthesis of short chain fatty acids.

6. Publications on the subject of dissertation

Publications in scientific journals

1. **Á. Mayer**, J. Rezessy-Szabó, Cs. Bognár - Á. Hoschke (2003): Research for creation of functional foods with bifidobacteria. Acta Alimentaria. **32**: (1):27-39
2. **Á. Mayer**, H. Seiler- S. Scherer (2003): Isolation of Bifidobacteria from Food and Human Faeces and Rapid Identification by Fourier Transform Infrared Spectroscopy. Annals of Microbiology. **53** (3): 299-313

Project report

1. Hoschke Á., Rezessy-Szabó J., Bujna E., **Mayer, Á.**, Bognár, Cs., Barna, Zs. (2000): Production and Evaluation of Functional Food Ingredients in Improving the Nutritional Quality of Food and Human Health. Final Report of IC-CT 96-1000.

Publications in conference proceedings

1. **MAYER Á.** (1998): Significance of Bifidobacteria in preservation of health (In Hungarian), National Scientific Student Conference of the Hungarian Scientific Society for Food Industry
2. **Mayer, Á.,** Rezessyné Szabó J., Bognár, Cs., Bujna, E., Hoschke Á. (1999): Isolation and Some Biological Properties of Human Intestinal Bifidobacteria. Food Microbiology and Food Safety into the next Millennium, Proceedings of the 17th International Committee on Food Microbiology and Hygiene, Veldhoven, The Netherlands,1999, pp.843-848.
3. **Mayer, Á.**, Schuster-Gajzágó I., Rezessy-Szabó J., Hoschke Á. (1999): Fermentation of Pea Flour by Bifidobacteria to Prepare Functional Food, Functional Foods- A new challenge for the food chemists Proceedings of the Euro Food Chem. X, Budapest, Hungary 1999, pp. 607-611.
4. Rezessy-Szabó J., **Mayer Á.**, Hoschke Á. (2003): Isolation, characterisation, cultivation of bifidobacteria and their implications in food technology. Flair Flow Workshop abstracts Functional Foods, probiotics:22-27

Presentations on scientific conferences

1. **MAYER Á**. (1999): Significance of Bifidobacteria in preservation of health (In Hungarian) National Scientific Student Conference, Gyöngyös, , First place
2. **Mayer, Á.,** Rezessyné Szabó J., Bognár Cs., Bujna E, Hoschke Á. (2000): Research on the establishment for the production of functional foods (In Hungarian). Lippay János-Vas Károly International Scientific Meeting, Budapest
3. **Mayer, Á**., Rezessyné Szabó J., Bognár Cs., Bujna E, Hoschke Á. (2000): Role of the probiotic *Bifidobacteria* and prebiotics supporting their growth in the preservation of health. (In Hungarian) Meeting of the Hungarian Society of Microbiology, Keszthely
4. **Mayer, Á**., Rezessy-Szabó J., Hoschke Á. (2001): Research for creation functional foods with bifidobacteria isolated from human source. Yearly gathering: Microbiology of the GI-tract, Bilthoven, The Netherlands

5. Rezessyné Szabó, J., **Mayer, Á.**, Bognár, Cs., Hoschke Á. (2001): Probiotics and their role in the prevention of the diseases of the colon. Enteric flora: from physiology to diseases. (In Hungarian) Meeting of the Hungarian Society of Gastroenterologists, Colon Section, Miskolc

Posters presented on scientific conferences

1. **Mayer, Á**., Bujna, E., Bognár, Cs., Rezessyné Szabó J. (1998): Morphological and biochemical characterization of *Bifidobacterium* species and their role in the preservation of health. (In Hungarian) Meeting of the Hungarian Society of Microbiology, Miskolc
2. **Mayer, Á**., Bujna, E., Bognár, Cs., Rezessyné Szabó J. (1998): Possibilities for the development of functional foods. (In Hungarian) Lippay János-Vas Károly International Scientific Meeting, Budapest
3. **Mayer, Á**., Seiler,H., Scherer, S. (2000): Identification of *Bifidobacteria* by Fourier Transformation Infrared Spectroscope (FTIR) (In Hungarian) Lippay János-Vas Károly International Scientific Meeting, Budapest
4. **Mayer, Á.**-Venema, K. (2002): Survival and mode of action of bifidobacteria from human origin in TNO's in vitro gastrointestinal tract models. Croatian, Hungarian and Slovenian Symposium on Industrial Microbiology and Microbial Ecology, Opatija, Croatia
5. **Mayer, Á**, Rezessy-Szabó J., Kondás B., Hoschke Á. (2003): *Bifidobacterium bifidum* B7.1 isolated from human origin is a promising probiotic strain. 1st FEMS Congress of the European Microbiologists, Ljubljana, Slovenia
6. **Mayer, Á**., Dücső, L., Mészáros, I., Rezessy-Szabó, J., Hoschke, Á. (2003): Mass Propagation and Immobilization of *Bifidobacteria.* 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary
7. **Mayer, Á**, Mészáros, I., Dücső, L., Rezessyné Szabó, J., Hoschke, Á. (2003): Formulation and preservation of probiotic bifidobacteria. (In Hungarian) Lippay János – Ormos Imre - Vas Károly International Scientific Meeting, Budapest
8. Kun, Sz., Rezessy-Szabó, J.M., **Mayer, Á.**, Nguyen, D.Q., Hoschke, Á. (2004): Cultivation of Probiotic *Bifidobacterium* Strains in Synthetic and Natural Media. 2nd Central European Congress of Food, Budapest, Hungary