DOCTORAL (PHD) DISSERTATION THESES

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ORGANOLEPTIC PROPERTIES AND PHYSIOLOGICAL EFFECT OF WHEY BASED PRO- AND PREBIOTIC FERMENTED MILK PRODUCTS

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1. **Background and objectives of the research**

Fermentation is one of the oldest methods for preservation, known to in the Old Testament, Abraham’s long life was connected to his regularly yogurt consumption without understanding the scientific basis of their effects. Nowadays there are a number of strong evidences about the beneficial effect on human health of using probiotics daily in an adequate number. Probiotics are known to have a long history of use, nowadays can be formulated in different types of form such as fermented milk products, drinks, cereals, meat products and also pharmaceuticals tablets. When the diverse collection of microbes, the human intestinal microbiota is disrupted it may become deleterious, to the host health is termed dysbiosis. One of the appropriate way for the bacterial reshape and colonization of the „forgotten organ” is the application of health-positive bacteria, such as the well known bifidobacteria and lactobacilli. Numerous bacterial species have been suggested to have probiotic effects, but strains from the genera of *Lactobacillus* and *Bifidobacterium* are added most commonly as probiotics to the variety of dairy and non-dairy products.

Yogurt and all dairy products can be the most natural media/food matrix for introduce probiotics to the human intestinal tract, thereby with added value, like a functional food. Functional foods seems to be the parting-line between food and medicine, they have gained much interest in nowadays due to their ability of health-promoting capacity. Should be a wise alternative to be consumed the health related component day by day as a part of a balanced diet. Milk and fermented dairy products are a rich source of beneficial compounds which have a good effect for the
digestive, gastrointestinal function, microflora and immunoregulation with a direct probiotic effect (interaction with the microflora) and with an indirect biogenic effect (production of the microbial metabolites) too. Fortunately, consumers demand for the consumption of these healthy, bio-defend functional foods are increasing nowadays due to the growing awareness about the impact of their diet on the health (primer prevention). Well-ballanced diet is a major focus of public health strategy aimed at maintaining the human health throughout life, and prevent several common disease which is closely related with the „western disorders” such as inflammatory bowel disease (IBD), Crohn-disease (CD), cancers, obesity, both type 1- and type 2-diabetes, polycystic ovarian syndrome (PCOS), bacterial vaginosis (BV) and asthma.

Whey has a number of functions which are beneficial to the health, good energy source, high content of essential amino acids and soluble vitamins also. The increasing amount of whey produced during dairy processes cause an increasing environmental protection problem, however it can be solved with a favourable opportunity by conversion of whey into value-added products.

The aim of my PhD research was to establish a new functional product of drinking yogurt supplemented with dairy by-products (whey), which due to its composition can be pro- or synbiotic, so it can have a health preserving effect by stabilizing the intestinal ecosystem. Furthermore, I investigated the manifestation of the physiological effect of LA-5 and BB-12 probiotics depending on the carrier (food-matrix or dietary supplement capsule).
Prior to the research, I formulated the following main objectives:

- Prepare the technological experiments in order to establish the future of the yogurt production. Investigation of microbiological, chemical and organoleptic characteristics as well as functional technological aspects of conventional, probiotic and synbiotic products made from milk and whey mixture after production and during the storage period. To determine the effect of pro- and prebiotics as well as whey supplementation on these properties, whether the probiotic counts meets the standards, and whether there is a significant change in the refrigerated storage of the products.

- Perform a simulated human digestion experiments to assess the extent to which beneficial bacteria survive during digestion. Comparison of conventional probiotic and synbiotic products made from milk and whey mixture and probiotic capsules used as a dietary supplement in terms of the extent to which the tested bacteria with physiologically beneficial properties reach their site of utilization in the colon in vitro.

- To evaluate the preconditions of the probiotic effect, to investigate the yoghurt starter culture and the ability of the tested probiotic strains to adhere to intestinal epithelial cells by in vitro hydrophobicity and adhesion test.

- Observation of the role of yogurt starter culture and probiotic strains in inflammatory processes in vitro.

- To investigate the extent to which conventional and probiotic products made from milk and probiotic capsules used as food supplements can alleviate the symptoms of artificially induced colitis in animal experiments.
2. Materials and methods

Yoghurt-type preparations were produced at the Technological Laboratory of Kaposvár University (legal predecessor of Hungarian University of Agriculture and Life Sciences, Kaposvár Campus) at the Food Science Research Institute of the National Center for Agricultural Research and Innovation, and at the INRAE Micalis Research Institute (Jouy-en Josas, France). For the preparation of the fermented dairy products I used skimmed milk powder and sweet whey powder (Obert Kft, Kaposvár), from which I prepared the ready-to-use milk and sweet whey by rehydration according to the product specification. I added 3% Frutafit Inulin IQ inulin (DP≥9) (Sensus, Roosendaal, The Netherlands) to the prebiotic-containing products. Yoghurts were treated with FD-DVS YF-L812 Yo-Flex (Chr. Hansen A/S, Horsholm, Denmark) with a multi-mixed starter culture (L. delbrueckii ssp. bulgaricus and S. thermophilus) and FD-DVS BB-12 and LA-5 (B. animalis spp. lactis and L. acidophilus) with probiotic culture (10^8 CFU / g) (Chr. Hansen A/S, Horsholm, Denmark). The Bonolact® Pro + biotic capsules (Hungarian Dairy Experimental Institute, Mosonmagyaróvár), B. animalis spp. lactis BB-12 and L. acidophilus LA-5 bacterial strains were lyophilized in a cellulose capsule in a TransMatrix™ protective cap package. One capsule contained 10^8 CFU of bacteria for both strains.

Technological assays

To check the change in the number of germs during the storage of the product, I performed microbiological plating tests on selective media corresponding to the given bacterial strain. Fourteen of the employees
and students of the Kaposvár University (legal predecessor of Hungarian University of Agriculture and Life Sciences, Kaposvár Campus) took part in the sensory evaluation. The participants in the critique acquired the characteristic properties of traditional natural yoghurt and drinking yoghurt in a preliminary sensory training, which was needed for later, accurate comparability. The pH change of drinking yoghurts during storage was monitored with a Testo 205 portable pH meter (Testo, Reutlingen, Germany). Color measurement was performed with a Minolta Chroma Meter CR-300 (Konica Minolta, Essex, UK). The measurement is based on the CIELAB color system, during which the values “L *”, “a *” and “b *” are registered. The curd strength of the product was tested with a Zwick Roell Z005 static destructive materials testing machine (Zwick Roell, Ulm, Germany). To examine the curd strength of the formulations, I used the maximum force peak at the first compression, 1st Fmax, First Maximum Force (N). The chemical composition of the samples was measured according to the following standards: “Milk, cream and condensed milk - Determination of dry matter content” MSZ EN ISO 6731; “Determination of the total protein content of milk” MSZ 12325-82; “Determination of the fat content of cream, sour milk, cream preparations and flavored milk products” MSZ 9602: 1984. The determination of lactose content was performed as described in MSZ 3725: 1984 “ Test methods for sour milk preparations”.

**In vitro human digestion experiments**

I performed the conventionally recognized Infogest in vitro digestion model study, accepted by several international research groups, as described by Minekus et al. (2014). I complemented the evaluation with a colon phase, in a batch culture model, for 24 hours. To assess the
interaction of the probiotic bacteria with the pathogenic ones more precisely, I also performed the same investigation with the presence of $10^6$ CFU/ml *E. coli* and $10^6$ CFU/ml *C. perfringens*.

**In vitro assays**
Carcinoma / adenocarcinoma cell lines from HT-29 and Caco-2 human colon tumors (ATCC HTB38, ATCC HTB37, LGC Standards, Molsheim, France) were used for adhesion and immune response studies. Cell lines were maintained and assays were performed according to the protocol provided. Plating and ELISA (Biolegend, San Diego, USA) were used to quantify adherent bacteria. For the hydrophobicity test, the microbial adhesion to solvents (MATS) was determined by the method of Vinderola and Reinheimer (2003).

**In vivo animal experiments**
A moderated dinitrobenzenesulfonic acid (DNBS) rodent model was used to induce moderate intestinal inflammation. For the experiment, 7-8 week old C57BL / 6 sterile mice were used at the INRAE Institute for Agricultural Research, Animal Husbandry and Experimental Facility (IERP, Jouy-en-Josas, France). Animals weighing approximately 20 g each were anesthetized intraperitoneally, then a 3.5 cm long catheter (Solomon Scientific, San Antonio, USA) was inserted directly into the colon and 100 mg/kg body weight of DNBS (Sigma-Aldrich Co., St. Louis, Missouri, USA) in 30% (v/v) ethanol-PBS (phosphate buffered saline) was delivered with a syringe (Sigma-Aldrich Co., St. Louis, Missouri, USA). The DNBS dose was 2 mg per mouse. Mice in the control group, in which intestinal inflammation was not induced, received ethanol-PBS following the same protocol. After 10 days, I introduced the tested bacteria / yoghurts / encapsulated formulation, or
PBS (control group). On one probation, I injected 200 µL of test substance intragastrically into the animal with $1 \times 10^8$ CFU of bacteria and continued for 10 days, this was the so-called feeding period. Artificial inflammation in the intestinal tract of the animals was reactivated 21 days after the first DNBS administration (healing period) by a second dose of 50 mg/kg DNBS.

After stunning, the animals were sacrificed by cervical dislocation, then the abdominal cavity was opened, the colon removed and cut lengthwise. Subsequently, in order to determine the macroscopic values, I evaluated the colonic damage by a scoring method. Dextran conjugated with fluorescent isothiocyanate (FITC-dextran 3000-5000 Da, Sigma-Aldrich Co., St. Louis, Missouri, USA) was used to detect intestinal permeability. Plasma FITC levels were determined with a fluorimeter equipped with a microplate reader (Tecan fluorescence microplate reader, Tecan, Lyon, France). Myeloperoxidase (MPO) activity (a marker of neutrophil infiltration) was measured from the distal colon using a modified version of the protocol described by Bradley et al. (1982).
3. Results

During the implementation of the tasks formulated in the objectives, first I examined the extent whether the use of probiotics (LA-5 and BB-12), inulin and whey changes the properties of the preparations compared to traditional yoghurt. In order to establish the future products, the formulations were also tested during a four-week refrigerated storage period, during I determined how their acidity, curd strength, CIELAB color coordinates, and human organoleptic characteristics change over time and relative to each other.

Subsequently, using in vitro Infogest human digestion model, I examined whether the probiotic bacteria reach their place of utilization in a sufficiently large number of germs, in the colon, and whether the effective germ count is maintained in the case of whey supplementation. Due to the interpretation of the food matrix effect, in addition to yogurt preparations, I also examined capsules containing probiotics in this regard.

One of the recognized aspects of the probiotic effect is the action of the immunomodulatory impact which, presupposes the adhesion and colonization of probiotic bacteria in the colon. Therefore, in the next step, I examined also the yogurt starter culture and probiotic culture too. I determined the in vitro hydrophobicity, the propensity to adhere to intestinal epithelial cells (HT-29, Caco-2), the adhesion of mucin, and its effect on the immune response (IL-8). Subsequently, in an in vivo animal experiment, I used a DNBS-induced mouse model of colitis to observe the effect of the studied microbes and the milk products prepared with them on colitis. Accordingly, I performed the following studies: weight
gain, evaluation of macroscopic scores, myeloperoxidase activity (MPO), and intestinal permeability assay (FITC).

Technological experiments
During the shelf life study, I found that the germ counts of the probiotic strains (LA-5 and BB-12) did not decrease below $10^8$ CFU / ml for either formulation during the four-week refrigerated storage period. The probiotic germ count of whey products was significantly higher than the dairy products ($P<0.05$). Both probiotic whey and synbiotic whey preparations differed significantly from each other ($P < 0.05$), the number of germs of probiotic yogurts was higher. The germ counts of the probiotic preparations containing 100% milk and the synbiotic preparations prepared with the addition of 3% inulin did not differ significantly ($P\geq0.05$), so I did not find that inulin had a positive effect in the case of dairy and whey products for the germ count during four weeks of refrigerated storage.

The pH of the formulations did not change significantly during storage ($P\geq0.05$). In the case of milk yogurt which containing only starter culture and in the case of whey products with probiotics, the pH value was slightly but significantly higher than the other yogurts ($P <0.05$). During refrigerated storage, I did not observe a significant change in color coordinates as a function of time for whey-containing and dairy products. However, when comparing the formulations, the color of the whey synbiotic product, supplemented with inulin, the L * color coordinate of the three color coordinates, which is the brightness factor was significantly different from the others ($P <0.05$), was slightly darker.

Curd strength was unchanged during refrigerated storage ($P\geq0.05$). Among the milk products, inulin synbiotic yoghurt differed
significantly (P <0.05) from the others, but in an unfavorable direction, while within whey products, the consistency of each preparation differed significantly from the other (P <0.05), and inulin had a favorable effect for curd strength. The protein content of whey products was significantly (P <0.05) lower than the dairy products, and products supplemented with 50% whey had a significantly lower (P <0.05) fat content. Based on the results of the sensory examination, I found that the color, aroma, taste and texture of the 100% milk-based preparations did not change as a result of storage, and there was no difference – except of the color – in the organoleptic properties of the preparations containing whey. Whey probiotic products were found to be too sour by the reviewers, in order to have more favorable sensory properties, the addition of flavors, aromas and fruit pulses is strongly recommended.

*In vitro human digestion experiments*

During the human digestion - regardless of the food matrix (yogurt or capsule) - the utilized probiotic was present in an amount of 4-5 log\text{10} CFU / ml by the end of the small intestinal section. In the gastric stage, during simulated human digestion, the two probiotics in both capsules and yogurt formulations showed an extremely good survival rate of nearly 90%. The protective effect of inulin was not observed in the gastrointestinal tract.

During the small intestinal stage, there was a continuing tendency for the two probiotics to be more resistant to digestive enzymes than the strains of the starter culture. The survival rates of LA-5 and BB-12 in the whey-containing medium were almost similar to those in the milk-only medium. BB-12 was still more stable in the milky medium and capsule during the gastric and colon stages than LA-5. The protective effect of inulin was not seen in the small intestine either. Resistance to the
unfavorable conditions of the digestive phases strongly depends on the particular bacterial strain, in my study it was more decisive than the presence or absence of food matrix, as robust probiotics behave favorably in all matrices.

I experienced an increase in BB-12 during the colon stage. In terms of pathogenic inhibition of the products, both probiotics alone and whey yogurt formulations were able to inhibit *C. Perfringens* growth by more than 80% during 24 h incubation, whereas none of the formulations was effective in inhibiting *E. coli* growth.

*In vitro experiments*

During the hydrophobicity experiments, I found that the hydrophobic nature and favorable electron acceptor properties of BB-12 suggest a good ability to adhere in the gut, which was also confirmed in the adhesion studies with the Caco-2 cell line and mucin. For the HT-29 cell line, LA-5 bound significantly higher (P<0.05) than BB-12 and *L. bulgaricus*.

In the immune response study, it was observed that the two probiotics (LA-5 and BB-12) and the starter culture strain, *L. bulgaricus* significantly reduced IL-8 secretion in TNF-α-stimulated HT-29 cells. However, the effect was absent when the bacteria of the starter culture were used together or in combination with probiotics.

*In vivo experiments*

During the *in vivo* animal experiments, the probiotic strains tested alone, without a food matrix, significantly alleviated the adverse effects of DNBS-induced colitis in mice. Strains BB-12 and LA-5 prevented weight loss, inhibited the development of macroscopic scores and inflammatory processes (MPO), and avoided a significant increase in
intestinal permeability (FITC). BB-12 and LA-5 probiotics in yogurt reduced the rate of macroscopic lesions and did not result in a significant increase in intestinal permeability in DNBS-treated mice.
4. Conclusions

- The physical, chemical and organoleptic properties of fermented products made from 50% whey and 50% milk raw materials differed from those made from milk-only products. The use of whey, due to its low dry matter content, resulted in the formation of a drinking yoghurt-type product. Inulin, at a rate of 3% (used as a prebiotic), had a positive effect on the rheological properties of whey products. Inulin, used also as a prebiotic, may be suitable for improving the consistency of whey-containing products.

- The probiotic germ count of the low-fat pro- and synbiotic yoghurt preparations prepared with whey supplementation remained above the recommended value, during 4 weeks of refrigerated storage, and the replacement of half of the milk with whey resulted in an increase in probiotic germ count, so 50% whey 50% milk is an appropriate medium to form and maintain a sufficient amount of probiotic CFU (BB-12 and LA-5) in yogurt.

- During the passage of yogurt through the gastrointestinal tract (based on the results of in vitro human digestion experiments), the effect of the whey matrix (50% whey-50% milk) on CFU did not differ from the milk matrix. It follows that the survival of probiotic bacteria (BB-12 and LA-5) was not affected by the application of whey.

- Resistance to the unfavorable conditions of the digestive phases strongly depends on the given bacterial strain, in my study it was more decisive than the presence or absence of the food matrix.
The used two probiotics (LA-5 and BB-12) proved to be extremely robust in this regard.

- The immune response inducing effect (IL-8) of *L. bulgaricus* strain present in the starter culture used requires further *in vitro* and *in vivo* studies. This bacterium is not a probiotic, however, the effect caused by it is only caused by bacteria that are considered a probiotic.

- The two probiotics together and the starter culture together with the two probiotics did not induce a significant immune response (IL-8), in contrast to the individual studies. Exploring the background of the antagonistic effect requires further investigation.

- In an *in vivo* rodent model of DNBS-induced colitis, probiotic cultures (LA-5 and BB-12) reduced the symptoms of the disease on their own, but this effect was less pronounced when co-administered with yogurt-like formulations.
5. New scientific results

1. The application of 3% inulin had a positive effect on curd texture of low fat (compared to normal yoghurt P<0.05) probiotic (LA-5 and BB-12, $10^8$ CFU/ml) yoghurt prepared from 50% whey and 50% milk and yoghurt starter culture (S. thermophilus + L. bulgaricus). During the four-week refrigerated storage period, the bacterial count of the probiotic cultures remained above the initial set value ($10^8$ CFU/ml), moreover the pH, the color coordinates, the odor, the consistency and taste of the yoghurt did not change significantly.

2. In simulated human digestion experiments, at the end of the small intestinal phase, the CFU of probiotics (LA-5 and BB-12) proved to be effective for therapeutic aims (4-5 log CFU/g) for all of the investigated products, i.e. lyophilized pharmacological capsule forms, yoghurts with probiotic (LA-5 and BB-12 $10^8$ CFU/ml) fermented with a starter culture (S. thermophilus+L. bulgaricus) made from 100% milk, yoghurt made from a mixture of 50% milk and 50% whey, and symbiotic yoghurt (3% inulin). Subsequently, resistance to adverse conditions in the digestive phases in vitro proved to be independent of the composition of food matrix, or presence or absence of food matrix.

3. In the in vitro immune response (IL-8) assay, the L. bulgaricus strain of the starter culture had an anti-inflammatory effect by reducing the secretion of IL-8 in the TNF-α-stimulated HT-29 cell line.
4. Conventional yoghurt made from 100% milk, coagulated with a yoghurt starter culture (S. thermophilus and L. bulgaricus); probiotic (LA-5 and BB-12 $10^8$ CFU/ml) yogurt; and LA-5 and BB-12 probiotic cultures in capsule form both alleviated some symptoms of DNBS-induced colitis in mice. They inhibited the development of macroscopic lesions and avoided a significant increase in intestinal permeability.
6. **Recommendations**

- It is worth to reviewing our idea of whey as a polluting by-product and considering it as an optional raw material with a high biological value and a positive physiological effect. However, in addition to its preferred composition, it has the disadvantage that it is rapidly degraded due to its high water content. One of the possible ways of utilizing whey is to produce various concentrated products by dehydration, but to achieve this, it is necessary to build an investment technological machine park. One solution to facilitate its fresh use would be to process it by fermentation directly at the point of origin of the by-product.

- Although the organoleptic properties of products supplemented with whey ingredients (sour taste) differed from those made only from milk ingredients, this can be improved by adding flavor and aroma clays and fruit pulps in order to gain a favorable consumer acceptance.

- The use of whey, due to its low dry matter content, can have an adverse effect on the texture of yogurt-like products. Inulin had a beneficial effect on the rheological properties of dairy products. Inulin used as a prebiotic may therefore be suitable for improving the consistency of whey-containing products.

- With the addition of whey, we have the opportunity to produce high value-added, functional, fermented low-fat dairy products, as it keeps the high probiotic CFU stable for 4 weeks during storage, moreover 50% whey-50% milk matrix resulted similar survival of probiotics in the intestinal tract than milk matrix.
• Further studies are needed to clarify the immune response-inducing effect of the applied starter culture on *L. bulgaricus* (IL-8), as well as the phenomenon that the two probiotics and the starter bacterium together with the two probiotics did not have a significant effect on the induction of the immune response, to obtain more information on strain-specific and dose-dependent effects.

• In an *in vivo* model of DNBS-induced colitis, probiotic cultures (LA-5 and BB-12) reduced the symptoms of the disease on their own, but this effect was less pronounced when co-administered with yogurt-like formulations. Further investigation is needed to determine whether this discrepancy was caused by micro- and macronutrients in the food matrix or by individual strains of the starter culture.

• Regarding static *in vitro* digestion models, in addition to being efficient, inexpensive, and easily reproducible, we need to consider a number of factors that require further development to be as close as possible to *in vivo* studies in their complexity. Dynamic and semi-dynamic *in vitro* models meet these requirements, however, dynamic digestion models require a large investment of equipment that cannot be implemented in all laboratories. An intermediate solution could be to use semi-dynamic models. It is important to state that although static *in vitro* models provide highly informative data, they are not a substitute for *in vivo* experiments. They mostly do not have the ability to model epithelial mucosa, have no interaction with the host immune system, and are not under neuroendocrine regulation.
The model used for the colon section can be further developed using a stool inoculum or a mixed bacterium model. Fecal inoculation would certainly model the colonic stage in a more complex way, but the complexity of the human microbiota greatly complicates the representative study, as the intestinal microbiota composition is influenced by many factors (age, lifestyle, geographical region, method of birth, breastfeeding).
7. Scientific papers and lectures on the subject of the dissertation

Peer-reviewed papers published in scientific journals

Gréta Pápai; Edgar Torres-Maravilla, Florian Chain, Éva Varga-Visi, Ottilia Antal, ZoltánNaár, Luis G. Bermúdez-Humarán, Philippe Langella, Rebeca Martín The administration matrix modifies the beneficial properties of a probiotic mix of *Bifidobacterium animalis subsp. lactis* BB-12 and *Lactobacillus acidophilus* LA-5. Probiotics and Antimicrobial Proteins 2020 August DOI:10.1007/s12602-020-09702-2 IF2020=3.53

Pápai, Gréta ; Szabó-Fodor, Judit ; Bóta, Brigitta ; Andrássyné, Baka Gabriella ; Antal, Otília Tamara ; Naár, Zoltán ; Vargáné, Visi Éva Savó hozzáadásának hatása pro- és szinbiotikus fermentált tejtermékek minőségére a tárolás során Élelmiszer-Tudomány Technológia 71: 2 pp. 27-33. , 7 p. (2017)


Judit, Szabó-Fodor ; András, Bónai ; Brigitta, Bóta ; Linda, Szommerné Egyed ; Ferenc, Lakatos ; Gréta, Pápai ; Attila, Zsolnai ; Róbert, Glávits ; Katalin, Horvatovich ; Melinda, Kovács Physiological Effects of Whey- and Milk-Based Probiotic Yogurt in Rats Polish Journal of Microbiology 66 : 4 pp. 483-490. , 8 p. (2017)
Conference paper and presentation:

Pápai, Gréta; Szabó-Fodor, Judit; Bóta, Brigitta; Naár, Zoltán; Vargáné, Visi Éva A savó mint melléktermék felhasználása, magas hozzáadott értékű, pre-és probiotikus funkcionális fermentált tejtermék előállítása céljából pp. 16-16. In: Gelencsér, É; Horváth, Zné; Rurik, I; Tömösközi, S (szerk.) Táplálkozástudományi Kutatások VII. PhD konferencia : program és előadás összefoglalók Budapest, Magyarország : Magyar Táplálkozástudományi Társaság, (2017) p. 23

Gréta, Pápai; András, Bónai; Brigitta, Bóta; Judit, Szabó-Fodor; Melinda, Kovács; Éva, Varga-Visi Whey is a promising media for producing a high microbial content pro- and synbiotic yogurt Journal of Probiotics And Health 3 : 3 pp. 80-80. , 1 p. (2015)

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Bónai, A ; Toldi, M ; Bóta, B ; Ölbeiné, Horvatovich K ; Bagóné, Vántus V ; Tuboly, T ; Kachlek, M ; Hafner, D ; Pápai, G ; Zsolnai, A