Special Issue Article "Clostridium Difficile Colitis" Short Communication

A Variety of Functional Characteristics of the Probiotic Lactobacillus Plantarum Inducia Contribute to the Prevention of Clostridioides Difficile Spore Germination

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ARTICLE INFO

Received Date: April 26, 2022
Accepted Date: May 16, 2022
Published Date: May 18, 2022

KEYWORDS

Clostridioides difficile
Spores
Germination
Lactobacillus plantarum INDUCIA

Citation for this article: Merle Rätsep, Liina Kuus, Kalle Kilk, Merle Muruvee, Sirje Kuusik, Oksana Gerulis, Mirjam Vallas and Epp Songisepp. A Variety of Functional Characteristics of the Probiotic Lactobacillus Plantarum Inducia Contribute to the Prevention of Clostridioides Difficile Spore Germination. SL Gastroenterology. 2022; 4(1):132

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ABSTRACT

Previously, we proved the possibility to prevent antimicrobial treatment-associated C. *difficile* infection and its recurrences by the antagonistic probiotic *L. plantarum* strain INDUCIA in synbiotic combination with xylitol.

The strain enhances natural cellular immunity and barrier function of gut mucosa via secretion of plantaricins, induction of cytokine IL-6, production of putrescine and increases the amount of lymphatic tissue in intestine. Strong antioxidative activity gives *L. plantarum* INDUCIA an extra protection, helping to counteract the oxidative stress imposed by bile acids or C. *difficile* associated inflammation-induced oxidative stress.

Here we aimed to clarify some mechanisms of action behind the ability of the strain to suppress the germination of C. *difficile*. The strain can endure bile acid-associated stress. INDUCIA-produced BSHs have specificity towards taurine-conjugated compounds, the result of which amongst others is chendeoxycholate, that can inhibit the germination of C. *difficile*. The antimicrobial activity of *L. plantarum* INDUCIA against C. *difficile* in some extent could also rely on secretion of plantaricins. Thus, a synergistic effect of a variety of functional characteristics of the probiotic *L. plantarum* INDUCIA could contribute to the prevention of C. *difficile* spore germination.

INTRODUCTION

Clostridioides difficile is an anaerobic gram-positive spore forming rod. Though being a part of the normal intestinal microbiota, toxigenic C. *difficile* strains are also the most common cause of Antibiotic-Associated Diarrhea (AAD) occurring during or after antibiotic treatment.

Besides being one of the most common causes of nosocomial infection, C. *difficile* is also a problem in nursing hospitals and retirement homes [1,2].

The C. *Difficile* Infection (CDI) of exogenous origin is initiated with the endospores released into the environment from patients with CDI and transmitted the fecal-oral route [3]. C. *difficile* spores pose a serious threat to elderly individuals with weakened immunity and decreased natural colonization resistance and dysbiosis [4]. In order to cause disease, ingested C. *difficile* spores must germinate [5]. In the intestine primary Bile Acids (BAs) and some secondary BAs stimulate the germination [6].

Primary BAs, synthesized de novo by the liver from cholesterol and conjugated to either taurine or glycine, are metabolized via multistep 7 a dehydroxylation pathway

into secondary BAs by the microbiota. First, conjugated BAs are hydrolyzed to free BAs and free glycine or taurine by microbial Bile Salt Hydrolase (BSH).

BSH activity is mainly observed in microbial species isolated from the Gastrointestinal Tract (GIT) [7]. BSH activity may act as protective mechanism against the toxicity of conjugated BAs and contribute to colonisation of the GIT [8]. However, wide variations exist among strains.

Lactobacillus plantarum is widely spread in nature on plant material and is utilised in food industry [9]. Large genome and high genomic diversity contribute the species the flexible metabolism and success in a variety of environments [10]. As a component of human intestinal microbiota, *L. plantarum* contributes to human health through immunomodulation of the host, competitive exclusion of pathogens, production of antimicrobial substances and antioxidants etc. These properties are also the basis for utilisation of *L. plantarum* as probiotic in functional foods and dietary supplements. Contradictory evidence exists regarding the efficacy of probiotics as adjunctive therapy for prevention or treatment of *Clostridioides difficile*-associated diarrhea.

Together with standard antibiotic treatment, probiotic efficacy in CDI prophylaxis has been tested in numerous clinical trials in form of monocultures or combinations of several strains and in different formulations (capsules, fermented food product) [11]. However, several questions are still open (e.g. the most effective formulation, duration of treatment and the optimal timing of probiotic administration). Information regarding the efficacy of probiotic combination with suitable prebiotic substances is also scarce.

Previously we repored a probiotic *Lactobacillus plantarum* strain DSM 21379 (acronym INDUCIA®) with antimicrobial activity that, in a synbiotic combination with xylitol in vitro completely stopped the spore germination of C. *difficile* [12]. The germination of toxigenic C. *difficile* was suppressed also in an experimental animal infection model, due to the pre-feeding and continuous administration of the symbiotic combination of *L. plantarum* INDUCIA and xylitol after C. *difficile* and ampicillin challenge.

Here we aimed to clarify some anti-C. *difficile* mechanisms of *L. plantarum* INDUCIA, particularly suppression of the germination of the pathogen.

MATERIAL AND METHODS

Probiotic strain

L. plantarum INDUCIA (DSM 21379) is of healty human origin. Its safety and functional properties have been tested *in vitro*, on experimental murine model and on volunteers [13]. *L. plantarum* INDUCIA possesses antioxidative activity and enhances natural cellular immunity and barrier function of gut mucosa via secretion of plantaricins, induction of cytokine IL-6, production of putrescine and increases the amount of lymphatic tissue in intestine. The functional properties and health effects are patented [13,14].

C. difficile strains

Clostridioides difficile strains reference strain VPI 10463 (ATCC 43255) and epidemic hypervirulent strain M 13042 were used in the study.

Bile acid deconjugastion assay

BA deconjugating ability of *L. plantarum* INDUCIA was tested in 0.05% w/v L-cysteine containing de Man Rogosa Sharpe (MRS; Oxoid, UK) broth with 4% w/v taurochenodeoxycholic acid (TCDCA; Sigma, USA).

The suspension 0.5 ml (McFarland 4; 1,2 x 10^9 cfu/ml) of the lactobacillus overnight culture was seeded into the reaction mixture. Sterlie MRS broth with TCDCA served as a control. After incubation (37°C, 24 hours), the supernatant was separated by centrifugation (2000 x g, 10 minutes). Concentration of TCDCA and chenodeoxyxcholic acid (CDCA) were assessed. The experiment was carried out in three parallels.

The 50 µl 10-fold diluted sample was mixed with 50 µl methanol and 50µl internal standard (50 µM [2H5] Phe). The samples were centrifuged 15 min 10000 × g, 100 µl of supernatant was diluted with 300 µl water and 5 µl injected into Shimadzu Prominence (Shimadzu Inc, Japan) HPLC and QTRAP 3200 (AB Sciex, USA) mass-spectrometry tandem. Flow rate was 0.2 ml/min and eluents: A – water with 0.1 % formic acid, B – methanol with 0.1 % formic acid. The first 2 min were isocratic flow of 5% eluent B, followed by rise to 90% eluent B in 5 min and final 10 min isocratic flow at 90% eluent B. The multiple reaction monitoring (MRM) transitions were 500.5/464 for TDCA and 375/357 for CDCA. Ionization was performed at 4500 V and 300°C, declustering potential was set to 25 V, collision energy 30 V.

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Effect of *L. plantarum* INDUCIA and xylitol combination on germination of *C. difficile* in the presence of bile acids

L. plantarum INDUCIA (final concentration 10^5 cfu/ml) was incubated anaerobically (anaerobic workstation CONCEPT 50, gases: 90%N: 5%CO2: 5% H2) for 24 hours in Brain Heart Infusion (BHI; Oxoid, UK) broth, BHI broth containing 5% xylitol (Merck KGaA, Germany) or mixture of xylitol with 4% w/v sodium taurocholate hydrate (TCA, Sigma-Aldrich, USA) or 4% w/v taurodeoxycholic acid (TDC, Sigma-Aldrich, USA). Spores of C. *difficile* were prepared by the alcohol shock method described previously [15]. The outgrowth of vegetative C. *difficile* cells from spores was verified by culturing. Spores (~10^2 cfu/ml) of C. *difficile* VPI strain were added to experimental mixtures and controls (sterile analogous mixtures), concentrated BHI broth in ratio 1:10 was added for additional nutrients and incubated for 48 hours of anaerobically. The viable counts (log_{10} cfu/ml) of INDUCIA and C. *difficile* were determined by the ten-fold serial dilutions at the beginning of the experiment, after 24 and 48 hours on MRS agar and Fastiodious Anaerobe Agar (FAA, Lab Ltd, UK) anaerobically. The experiment was carried out in triplicate.

Effect of *L. plantarum* INDUCIA supernatant with bile acids on germination of *C. difficile*

The experiment aimed to evaluate the effect of TCDCA on the viability of INDUCIA; the antimicrobial effect of INDUCIA and TCDCA combination on C. *difficile* germination and the nature of the putative plantaricin.

L. plantarum INDUCIA (final concentration 10^5 cfu/ml) was incubated anaerobically for 24 hours in BHI broth (Biolife, Italy) and in BHI broth containing 4% w/v TCDCA (Sigma, USA). The effect of TCDCA on the viability of INDUCIA was evaluated as described above.

From half of the BHI - TCDCA mixture, the Cell Free Supernatant (CFS) was collected by centrifugation (3000 g/ 10 min) and filter-sterilized (0.2µm, Sartorius Stedim Biotech, Germany).

For evaluating the nature of the putative plantaricin, *L. plantarum* INDUCIA was incubated for 24 hours in BHI broth containing 5% xylitol (Sigma-Aldrich, EU) and cell-free supernatant was obtained as describe above.

The plantaricin in CFS was inactivated by heat treatment (100°C, 30 minutes) or with 250 U/ml trypsin (Sigma, USA). After trypsin treatment, samples were incubated at 37°C for 2 hours. The enzyme was inactivated by heating to 100°C for 3 minutes. Spores (10^2 cfu/ml) of C. *difficile* strain M were added to experimental mixtures and controls (BHI with xylitol) and incubated for 48 hours anaerobically. The counts of C. *difficile* were evaluated as described above. The experiment was carried out in three parallels and in triplicate.

Effect of xyltol and glucose on the metabolite profile of *L. plantarum* INDUCIA

L. plantarum INDUCIA was grown for 24hours on MRS agar anaerobically (Anaerobic workstation CONCEPT 50, gases: 90%N: 5%CO2: 5% H_2) and microaerobically (10% CO_2).

A cell suspension (in final concentration McFarland 0.5 turbidity standard, $1.5x10^6$ cfu/ml) was prepared into the modified MRS broth without triammonium-citrate and sodium-acetate (pH 7.2), containing either 20g/L glucose or xylitol and incubated anaerobically and microaerobically for 24 h and 48 h. In the case of anaerobic environment, the modified MRS broth was beforehand reduced for 24 hours. The supernatant was separated by centrifugation (3000 g/ 10 min) and filter sterilised (0.2µm Sartorius Stedim Biotech, Germany). The experiment was carried out in three parallels.

Short chain fatty acids, lactic acid and ethanol were determined by gas chromatograph (Agilent 6890A) equipped with Flame Ionization Detector (FID) and capillary column CP-Wax 52 CB (30 m x 0.25 mm, 0.25 µm). Column temperature programm was 75°C 1 min hold, 10°C/min to 115°C 3 min hold, 20°C/min to 190°C 4 min hold and 30°C/min to 200°C 1 min hold. Temperature of the FID detector was 280°C. Injection was performed at 200°C and injection volume was 1µl with split ratio 10:1. Air and hydrogen were used as detector gases with flow rates 300 ml/min and 30 ml/min, respectively. Nitrogen was used as make-up gas with a flow rate 20 ml/min.

STATISTICAL ANALYSIS

Descriptive statistics was presented as means and standard deviations. Differences in means between study groups were tested using independent samples t-test. Differences were considered statistically significant when $p<0.05$. Statistical analyses were performed using R version 3.2.3 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Bile acid deconjugastion assay

TCDCA was degraded by INDUCIA to CDCA: an inverse change in TCDCA and CDCA concentrations in both CFS and in cell suspension was found after incubating *L. plantarum* INDUCIA with 4% TCDCA (Table 1).

Table 1: Changes (% and SD) in the levels of taurochenodeoxycholic acid and chenodeoxycholic acid in growth media. The change is presented as relative to the level present in media prior addition of *L. plantarum* INDUCIA.

	TCDCA[a]	CDCA[b]
Supernatant	(99±4)	(149±7)
Cells[c]	(84±22)	(126±54)

[a]TCDCA - taurochenodeoxycholic acid; [b]CDCA - chenodeoxy-cholic acid; [c]cells - medium with cell

Effect of *L. plantarum* INDUCIA and xylitol combination on germination of *C. difficile* in the presence of bile acids

To mimic in vitro the situation in gut environment, the effect of *L. plantarum* INDUCIA in synbiotic combination with xylitol to germination of C. *difficile* in the presence of bile acids was tested.

In BHI preincubated with INDUCIA, the C. *difficile* reference strain VPI 10463 germination was delayed until 24 h (Table 2). Preincubation of INDUCIA with xylitol or in mixture of xylitol-TDC or xylitol-TCA fully inhibited C. *difficile* germination. In controls the suppression of C. *difficile* germination was not detected for 48 hours.

Table 2: Vegetative cells (average and SD; log_{10} CFU/ml) of C. *difficile* reference strain VPI 10463 after germination of spores at the presence of *L. plantarum* INDUCIA, xylitol, sodium taurocholate hydrate and taurodeoxycholic acid in growth environment.

Mixture	Control			Experimental [a]		
	0 h	24 h	48 h	0 h	24 h	48 h
BHI	2.55 ± 0.74	5.80±0.70	7.43±0.03	nd	nd	7.45±0.05
BHI + 5% xylitol	2.40±0.75	7.07±0.63	7.43±1.36	nd	nd	nd
BHI + 5% xylitol + 4% TCA [b]	3.02±0.76	7.60±0.60	7.76±0.48	nd	nd	nd
BHI +5% xylitol +4% TDC [c]	3.38±0.08	7.73±0.84	7.67±0.06	nd	nd	nd

[a]Experimental - preincubated with L. plantarum INDUCIA
[b]TCA - sodium taurocholate hydrate
[c]TDC - taurodeoxycholic acid

dnd- not detectable (below the detection limit 1.00 log10 CFU/ml).

The viablitiy of *L. plantarum* INDUCIA was not negatively affected by the presence of 4% of TCA or TDC in the growth environment, reaching during 48 hours of incubation in BHI to 8.31 ± 0.01 log10 cfu/ml; in BHI with xylitol to 8.78 ± 0.35 log10 cfu/ml, in BHI with xylitol and TCA to 8.51 ± 0.53 log10 cfu/ml and in BHI with xylitol and TDC to 8.25 ± 0.26 log10 cfu/ml.

Effect of *L. plantarum* INDUCIA CFS with bile acids on germination of *C. difficile*

The presence of 4% of TCDCA in growth environment had no negative impact on the viability of *L. plantarum* INDUCIA (Table 3). During 48 hours of incubation, the viable counts reached in BHI to 7.67 ± 0.09 log_{10} cfu/ml and to 7.35 ± 0.14 log_{10} cfu/ml in BHI with TCDCA.

Table 3: Viable counts of *L. plantarum* INDUCIA (average and SD; log_{10} CFU/ml) in the presence of TCDCA.

Mixture	Viable counts of INDUCIA		
	0 h	24 h	48 h
BHI	5.41 ± 0.08	7.56 ± 0.07	7.67 ± 0.09
BHI +4% TCDCA[a]	5.24 ± 0.1	6.73 ± 0.1	7.35 ± 0.14

[a]TCDCA - taurocenodeoxycholic acid

In BHI preincubated with *L. plantarum* INDUCIA, the germination of C. *difficile* hypervirulent strain M 13042 was delayed until 24 hours (Table 4). In contrary to the reference strain VPI 10463 (Table 2), the germination was significantly disturbed for 48 hours (control 8.36±0.23 log10 cfu/ml vs. 2.36±0.03 log_{10} cfu/ml, $p < 0.0001$).

The C. *difficile* germination was fully inhibited in TCDCA-INDUCIA cell suspension mixture and in the TCDCA-CFS mixture after 24 hours. The pH of the CFS of *L. plantarum* INDUCIA in BHI broth of was 6.

Both the heat and the trypsin treatment CFS had significant disruption ($p < 0.0001$) to C. *difficile* germination throughout the experiment in comparison with the control (Table 4).

Effec of xyltol and glucose on the metabolite profile of *L. plantarum* INDUCIA

L. plantarum INDUCIA grew equally well microaerobically and anaerobically in the presence of glucose, the viable count reaching during 48 hours of incubation respectively to

8.890±0.055 log10 cfu/ml and 8.863 ±0.100 log_{10} cfu/ml. Lactic acid was the predominant end product of glucose fermentation (Table 5). Additionally, traces of acetate and after first 24 hours of incubation also traces ethanol were detected.

Table 4: Vegetative cells (average and SD; log_{10} cfu/ml) of C. *difficile* epidemic hypervirulent strain M 13042 after germination of spores at the presence of different factors in growth environment (*L. plantarum* INDUCIA cells, cell-free supernatant, xylitol, or taurocenodeoxycholic).

Mixture	Control			Experimental[a]		
	0 h	24 h	48 h	0 h	24 h	48 h
BHI	2.15±0.13	6.57±0.26	8.36±0.23	nd[a]	nd	2.36±0.03
BHI + 4% TCDCA[b], CFS[c]	2.29±0.33	7.59±0.22	7.77±0.44	1.99±0.04	nd	nd
BHI +4% TCDCA, cells	2.29±0.33	7.59±0.22	7.77±0.44	nd	nd	nd
BHI + 5% xylitol, heat treated	2.16±0.23	7.56±0.15	7.68±0.10	1.78±0.08	1.67±0.23	1.04±0.05
BHI + 5% xylitol, trypsin treated	2.07±0.04	7.53±0.15	7.62±0.09	1.55±0.10	nd	1.16±0.12

[a]Experimental - preincubated with L. plantarum INDUCIA
[b]TCDCA - taurochenodeoxycholic acid
[c]CFS - cell free supernatant
[d]nd- not detectable (below the detection limit 1.00 log10 CFU/ml)

Table 5: Production of lactic and acetic acids (average and SD; mg/ml) by *L. plantarum* INDUCIA from glucose in anaerobic and microaerobic milieu.

Time	Environment	Lactic acid	Acetic acid
24 h	Glc AN	7.866 ± 0.007	0.009 ± 0.005
48 h	Glc AN	8.869 ± 0.813	0.015 ± 0.022
24 h	Glc MA	7.319 ± 0.143	0.015 ± 0.007
48 h	Glc MA	8.999 ± 0.791	0.024 ± 0.022

MA: Microaerobic; AN: Anaerobic; Glc: Glucose

In the modified MRS broth, the viability of the strain under anaerobic cultivation was not negatively affected by the presence of xylitol, though the cell proliferation was in standstill. Under microaerobic cultivation, nearly 1-log decrease in viability occurred after 24 hours. Only acetic acid was found in detectable amounts.

DISCUSSION

Previously, we proved the possibility to prevent antimicrobial treatment-associated C. *difficile* infection and its recurrences by the antagonistic probiotic *L. plantarum* strain INDUCIA in synbiotic combination with xylitol [12]. Here we aimed to clarify some mechanisms of action behind the ability of the strain to suppress the germination and outgrowth of C. *difficile*.

Preservation and restoration of the microbial diversity represents one of the strategies for prophylaxis or treatment of CDI, as the alteration of intestinal microbiota and diminished microbial diversity is considered one of risk factors for CDI [16]. Intestinal lactobacilli pose an important factor in maintaining colonization resistance against C. *difficile*. Allegedly, particular species may be more effective than others. For instance, during the evaluation of intestinal lactobacilli of Estonian and Norwegian patients with AAD, the number of species Lactobacillus plantarum was found to be in low in C. difficile positive patients and more common in C. *difficile* negative subjects [17]. Thus, *L. plantarum* INDUCIA may have some species-specific advantage as an anti-C. *difficile* probiotic. As INDUCIA survives gastrointestinal passage and grows well in anaerobic milieu, it can compete with the pathogen for nutrients and adhesion sites, thus helping to restore the colonization resistance. In C. *difficile* infection model, the pre-feeding and continuous subsequent supplementation with the symbiotic combination of INDUCIA and xylitol to hamsters infected with C difficile spores increased the survival rate up to 78% (in comparison with 13% in controls) together with lower level of colonization with toxigenic C. *difficile* [12].

Though mostly temporary colonizers, probiotics help to restore normal gut microbiota by producing a variety of biocative compounds, that create unfavorable environment for C. *difficile* or help to restore CDI induced damages in GIT. One of the anti-C. *difficile* mechanisms of INDUCIA in situ could be due to reducing the luminal pH through organic acid and short chain fatty acid (SCFA) production, which in turn promotes calcium absorption. Calcium ions in the GIT facilitate C. *difficile* germination, colonization, and pathogenesis [18].

SCFA also play essential role in the integrity of mucosa. Aditionally, xylitol, administered simultaneously with the probiotic and reaching the lower intestine, contributes to the

increase of the SCFA concentration through positive change in fecal microbial population [19-21].

Besides SCFA, other microbial metabolites are important for enhancement of mucosal barrier. Different positive effects of polyamines (putrescine, spermidine and spermine) have been described including suppression of inflammation [22-25]. Lactobacilli produce polyamines through decarboxylation of amino acids [26]. *L. plantarum* INDUCIA produces in vitro putrescine, the precursor for spermidine and spermine and in situ into food matrix (cheese) [13]. However, the effect of a probiotic on polyamine content in GIT is strain specific. For example, Sepp and co-workers [27] reported a negative correlation between lactobacilli count and urine polyamines after adminsitration of L. fermentum ME-3. On the other hand, the presence of *L. plantarum* INDUCIA increased the concentration of polyamines, especially putrescine in intestinal lumen, thus contributing to the improvement of intestinal barrier function [13].

Our in vitro data suggest, that *L. plantarum* INDUCIA can endure BAs - associated stress. Next, INDUCIA possesses strong antioxidative activity both in aqueous and lipid environments [13]. This property gives the strain an extra protection during gastrointestinal passage and helps to counteract the oxidative stress imposed by BAs or C. difficile associated inflammation-induced oxidative stress.

In human bile, glycine-conjugated BAs prevail, and taurine-conjugated BAs are more common in the case of Western diet associated with high levels of animal protein [28-29]. It has been demonstrated, that the BSHs of most Lactobacillus spp strains express higher substrate specificity towards glycine-conjugated BAs [29-31].

Herein, on the example of TCDCA, we demonstrated that INDUCIA is capable to hydrolyse taurine - conjugated compounds, as TCDCA was degraded to CDCA by the strain. In contrary to cholate and cholate derviates (taurocholate and deoxycholate), that act as germinants for C. *difficile*, CDCA inhibits the germination and even blocks other BAs -mediated germination of C. *difficile* [6,32]. Thus, the specificity of INDUCIA-produced BSHs towards taurine – conjugated BAs might be another mechanism behind the antimicrobial effec against C difficile. Yet, the exact substrate specificity of *L. plantarum* INDUCIA BSHs needs further clarification.

Next, the genome of *L. plantarum* INDUCIA harbours IIb plantaricins EF and K and plantaricin MG encoding genes (unpublished data). The distinctive feature of the latter (besides the broad antibacterial activity, heat and pH stability) is its ability to inhibit not only vegetative cells of Gram-positive bacteria (incl. C. *difficile*) but also the germination of their spores [33]. Thus, the antimicrobial activity of *L. plantarum* INDUCIA against C. *difficile* in some extent could rely on secretion of plantaricins [13]. Most bacteriocins produced by lactic acid bacteria are thermostable and sensitive to proteolytic enzymes [34]. The heat and trypsin treatments of INDUCIA CFS resulted in some loss of activity, that hints their proteinaceous nature. However, the expression of plantaricins by INDUCIA and their nature needs further investigation.

CONCLUSION

A synergistic effect of a variety of functional characteristics of the probiotic Lactobacillus plantarum INDUCIA could contribute to the prevention of Clostridioides difficile spore germination and outgrowth: antioxidative and BSH activity, production of organic acids, plantaricins and polyamines.

ACKNOWLEDGMENTS

The research was co-financed by EU Regional Development Fund under Projects EU30002 and EU 48686 of the BioCC OÜ.

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