

ORIGINAL ARTICLE

GUT MICROBIOTA ALTERATIONS BY NUTRITIONAL SUPPLEMENT IMUREGEN

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Summary

The gut microbiota is one of the modulators influencing its host's development, metabolism, as well as immunological, psychological, and cognitive abilities. The gut microbiota consortium influences enteroendocrine regulation, neurohormonal regulation, as well as natural immune regulation. Disruptions occurring in life can lead to dysbiosis that in turn influences the host homeostasis and/or disease. Targeted modulation of microbiota composition thus appears to be an appropriate intervention strategy in cases of certain specific health problems. Here, we demonstrate that application of the nutritional supplement Imuregen, which is a natural immune booster, modulates the *Bacteroidetes/Firmicutes* ratio in favor of the *Bacteroidetes* genera and causes no pathological changes to intestinal epithelium.

Key words: microbiome; microbiota; Imuregen; immune modulation

INTRODUCTION

The gastrointestinal tract of mammals is colonized by myriad microbes that are generally and collectively termed the microbiota. For this reason, the mammalian, and perhaps all multicellular organisms, may be considered metaorganisms. The microbiota and its host affect one another through their very existences, by products of their metabolisms, as well as through bioactive molecules that are produced as a consequence of their mutual interaction. The microbiota plays a fundamental role in modulating host physiology at large, even as its host affects the ecology of the microbiota and its species diversity. Disruption of the host's natural relationships with its microbiota can lead to serious host health problems.

The formation of that metaorganism begins at birth. A vaginally delivered baby acquires a spectrum of bacteria resembling its mother's vaginal microbiota and dominated by the genera *Lactobacillus*, *Prevotella*, and *Sneathia*. Within the microbiota of babies delivered by Cesarean section the dominant genera are *Staphylococcus*,

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Corynebacterium, and *Propionibacterium*, which spectrum is similar to that present on the skin (1). The consortium of the host and its microbiota together dictates the health status of the metaorganism. The modulatory effect of a mammal's gut microbiome has been documented in relation to the immune system's maturation and its function (2), orchestration of microbiota–gut–brain communication (3-5), and influencing the origin and therapy of cancers (6,7). Knowledge as to how the microbiota interacts with the host and its constituent species with one another nevertheless remains elusive (8). Moreover, the practice of exerting targeted influence on microbiome species composition is still in its infancy.

What is generally known is that antibiotics have perturbation effects on the human gut microbiome. A majority of antibiotics (including macrolides (9), clarithromycin (10), and vancomycin (11)) reduce the numbers of *Firmicutes* and *Actinobacteria* while upregulating numbers from the genus *Bacteroides* and phylum *Proteobacteria*. Ciprofloxacin and clindamycin have similar effects on gut microbiota (12,13). Diet, however, is still the dominant modulator of gut microbiota. The literature documents that diet strongly modifies that microbiota, which in turn has a profound impact on the overall health of its host (14,15). In recent decades, the status of human gut microbiota has been strongly influenced by modern food processing technology. For example, such noncaloric artificial sweeteners as saccharin, sucralose, and aspartame change the composition of gut microbiota in ways that are linked to host susceptibility to metabolic abnormalities and glucose intolerance (16). Relatively low concentrations of commonly used emulsifiers, such as carboxymethyl cellulose and polysorbate 80, have been shown to induce low-grade inflammation and metabolic syndrome in mice, thereby promoting robust colitis in mice predisposed to this disorder. Emulsifier-induced metabolic syndrome has been associated with changed gut microbiota species composition and increased pro-inflammatory potential (17,18). It has also been well-documented that consumption of an energy-dense diet changes the gut microbial community and induces low-grade inflammation of bowel tissues in various animal models (19–21). Moreover, an energy-dense diet-induced shift in gut microbiome may disrupt vagal gut–brain communication, thereby resulting in microglia activation and increased body fat accumulation (22).

All the facts mentioned above demonstrate the need to intervene very carefully into the human nutritional composition in order to minimize adverse effects of technologically prepared nutrition and of civilization's stressors. Imuregen, a natural nutritional supplement that is widely distributed across the world, has been tested using various laboratory model systems as well as in different clinical situations (23). All the data collected through several decades have demonstrated that Imuregen is harmless, is non-genotoxic, is not mitogenic, has no side effects, and is beneficial for the users. Nevertheless, there is still no biological understanding as to why Imuregen has such clinical effects. One of the possibilities might relate to its influence on gut microbiota composition, which can gently modulate the physiological processes of the host.

Here, we document the alteration of murine gut microbiota induced by the natural immune booster Imuregen. We then discuss possible impacts of this alteration on several clinically significant situations.

MATERIAL AND METHODS

Material

Tissue extract: The substance of the Imuregen preparation was a kind gift from Uniregen, Ltd. Nachod, Czech Republic. The substance was stored in darkness at room temperature until used for individual experiments.

Animals: Female specific pathogen-free BALB/c mice were purchased from Velaz (Unetice, Czech Republic) and were used at 6–8 weeks of age. Mice were placed in sterile cages. Air temperature was stabilized at 22 ± 2 °C. The light regime was 12 h light and 12 h darkness. Experiments on mice were conducted under supervision of the institution's animal unit and were approved by the Animal Care and Use Committee of the Faculty of Military Health Sciences, University of Defense, Hradec Kralove, Czech Republic.

Application of Imuregen

Imuregen was administered to mice through the drinking regime. Imuregen was administered to mice in water that had been treated to reduce microbial contamination. Imuregen was diluted to concentration of 10 mg/l.

The average daily consumption of water for an adult 25 g mouse was around 5 ml. This volume contains 50 µg of the substance per mouse per day (1 mg substance per 100 ml). Control mice had access ad libitum to potable, uncontaminated drinking water without any added substances. Fresh liquid substance of Imuregen was prepared daily during morning. The water delivery system was checked daily to ensure proper function. Care was taken to be sure water delivery systems did not leak, particularly when cages were moved during cleaning or transport. To minimize the risk of cross-contamination, care was taken that water bottles were not interchanged between groups of mice. Such established drinking scheme was followed for 30 days.

Tissue collection and processing

Mice and humans are quite similar in their physiologies and anatomical structures, and this is one of the reasons why a mouse model was used in our studies. Imuregen-treated and control mice were maintained on a standard diet, and sacrificed 30 days post-gavage. Mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation. Sections and contents of the ileum and proximal colon were collected separately and placed into Eppendorf tubes. Tissues were stored at −80 °C until further use for analysis.

Bactericidal tests

Escherichia coli CCM 3954 and *Staphylococcus aureus* CCM 4223 were used for testing bactericidal activity. Bacterial strain suspensions were inoculated onto blood agar plates evenly distributing OD = 1.0 or diluted 10 times (OD = 0.1). Imuregen was dissolved in deionized H₂O and applied at predetermined concentrations onto sterile Whatman paper discs, which were placed immediately onto the seeded culture plates. Alternatively, the Imuregen was dissolved in deionized H₂O to concentration 10 mg/ml. The suspension of bacteria *Francisella tularensis* LVS ATCC 29684, *Escherichia coli* CCM 3954, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* CCM 3955, and *Staphylococcus aureus* CCM 4223 at concentration OD = 1 were dissolved in saline and Imuregen according to the scheme below (Fig. 1).

Histology of tissue samples

Tissues for histological examination were fixed, embedded in paraffin, stained by hematoxylin-eosin and by stain for acid mucopolysaccharides following the method of Hale-Müller. During examination by light microscopy, the height of villi was evaluated by computer morphometry.

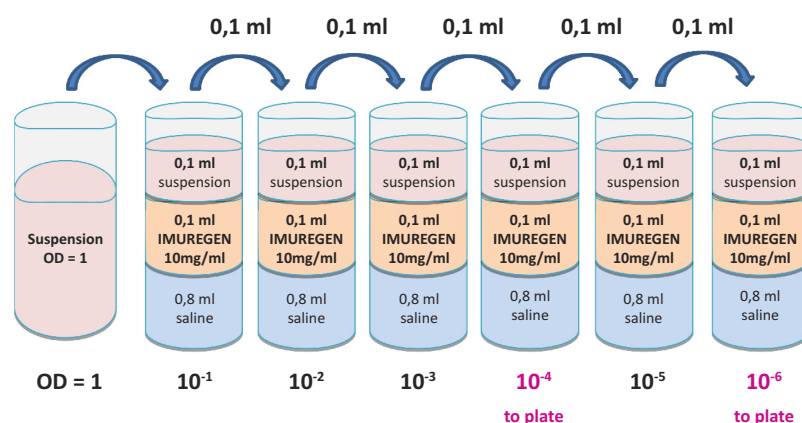


Figure 1. Scheme of preparing bacterial suspension for alternative testing of bactericidal ability of Imuregen.

Microbiota composition

Microbiota composition was determined in ileal and cecal contents. The contents were homogenized in a MagNALyzer (Roche). Following homogenization, the DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions and the DNA concentration was determined

spectrophotometrically. DNA samples were diluted to 5 ng/ml and were used as template in polymerase chain reaction (PCR) with forward primer 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-MID-GT-CCTACGGGNGGCWGCAG-3' and reverse primer 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-MID-GT GACTACHVGGGTATCTAATCC-3'.

MIDs shown above represent different sequences 5, 6, 7, or 9 base pairs in length that were used to identify individual samples within the sequencing groups. PCR amplification was performed using a HotStarTaq *Plus* Master Mix kit (Qiagen) and the resulting PCR products were purified using AMPure beads (Beckman Coulter, Prague, Czech Republic). In the next step, the concentration of PCR products was determined spectrophotometrically, the DNA was diluted to 100 ng/μl, and groups of 14 PCR products with different MID sequences were indexed with the same indices using Nextera XT Index Kit (Illumina) while following the manufacturer's instructions. Prior to sequencing, the concentration of differently indexed samples was determined using a KAPA Library Quantification Complete kit (Kapa Biosystems). All indexed samples were diluted to 4 ng/μl and 20 pM phiX DNA was added to final concentration of 5% (v/v). Sequencing was performed using a MiSeq Reagent Kit v3 and MiSeq apparatus (Illumina) according to the manufacturer's instructions. The FASTQ files generated after quality trimming were uploaded into QIIME software. Forward and reverse sequences were joined with minimum 8 bp overlap. In the next step, chimeric sequences were predicted by the slayer algorithm implemented in QIIME and excluded from subsequent analysis. The resulting sequences were then classified by RDP Seqmatch with an OTU (operational taxonomic units) discrimination level set to 97%.

Statistical analysis

Data for all variables were normally distributed and in most cases were evaluated as means \pm SD. Statistical significance was determined by (un)paired Student's t-test. Statistical significance was established when $p < 0.05$.

RESULTS

Bactericidal activity of the Imuregen

To test the possible direct effect of Imuregen on Gram-negative and Gram-positive bacteria, we used two generally known bacterial genera: *Escherichia coli*, representing Gram-negative bacteria, and *Staphylococcus aureus*, representing Gram-positive bacteria. Three independent experiments were carried out. Imuregen was dissolved in deionized H₂O. Ten-microliter samples containing 100 μg/ml, 10 μg/ml, 1.0 μg/ml, and 0.1 μg/ml of Imuregen were applied to sterile Whatman paper discs and used on conducting Experiment 1. The experimental doses of Imuregen had no effect on bacterial growth on the plates. Experiment 2 was conducted with 1,000 times greater doses of Imuregen. Similarly to Experiment 1, no visible effect was observed on growth of the bacterial load on the plates (Fig. 2).

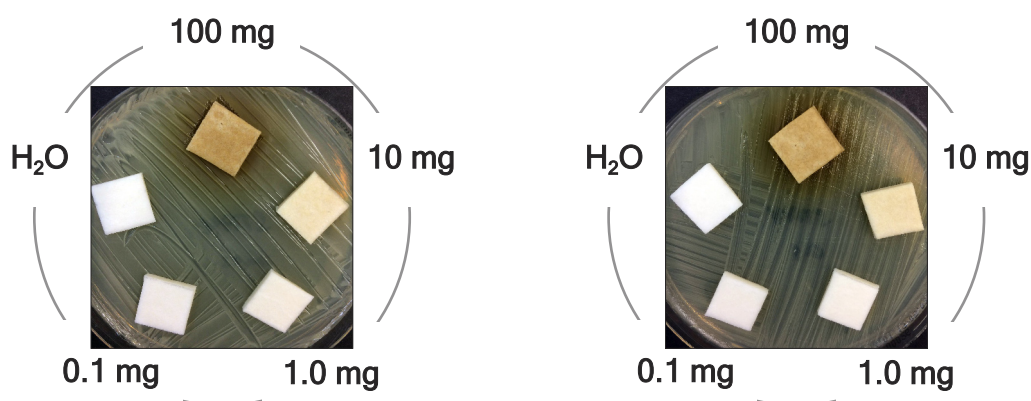


Figure 2. Example of results from Experiment 2. Plates seeded with *Staphylococcus aureus* CCM 4223 and cultivated 48 h with Whatman paper discs filled with Imuregen at indicated concentrations. Left plate was seeded with suspension OD = 1.0, the right plate with suspension diluted 10 times (example is of Experiment 2 results).

The experiment utilized decimal dilutions of bacterial suspensions. It revealed insignificant effect of Imuregen on the representative of Gram-negative bacteria, *Escherichia coli*. The Gram-positive *Staphylococcus aureus* was completely without any effect (Fig. 3 and Table 1).

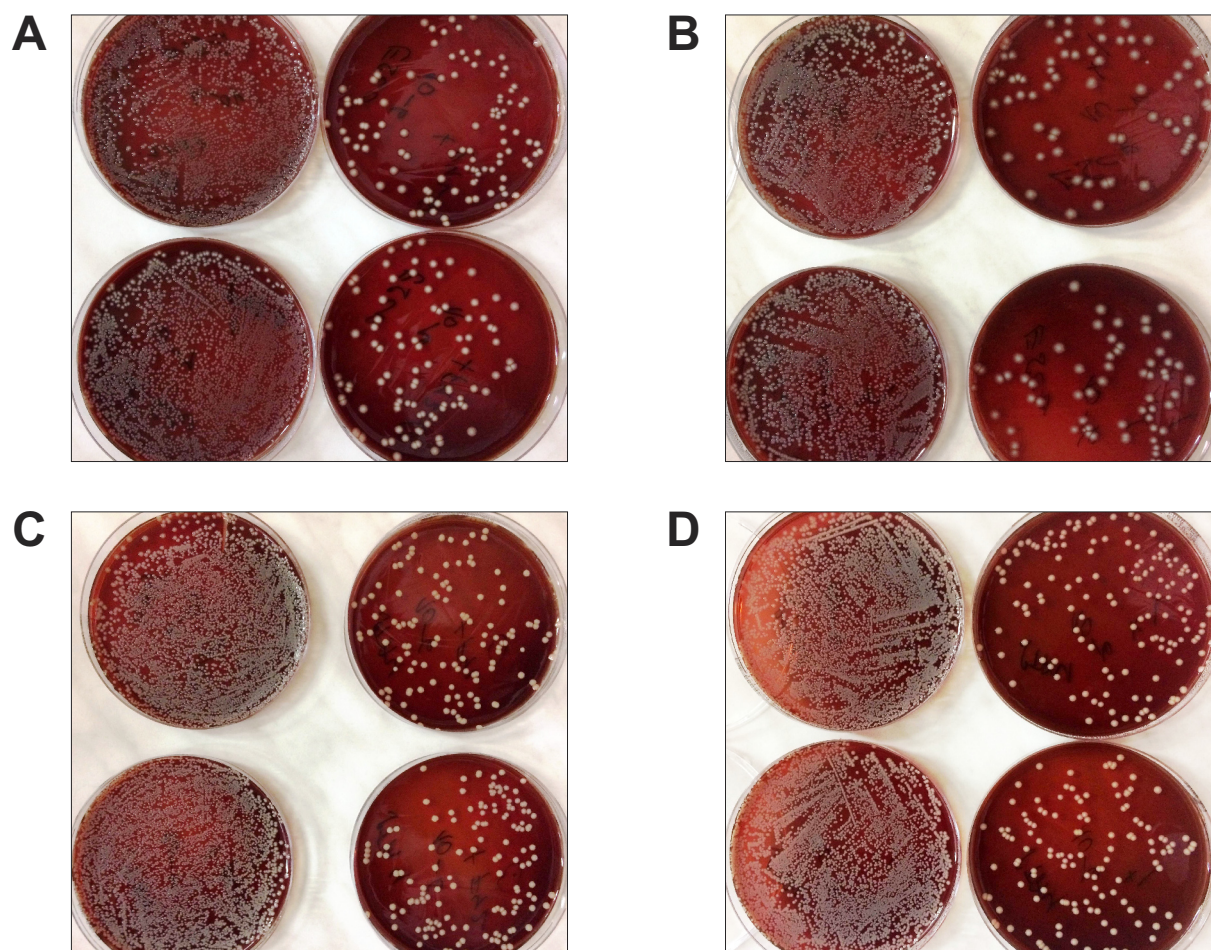


Figure 3. Plates demonstrating the result of the experiment conducted in accordance with the alternative protocol. The Imuregen was dissolved in deionized H₂O to concentration 10 mg/ml. The suspensions of bacteria *Escherichia coli* CCM 3954 (A, B) and *Staphylococcus aureus* CCM 4223 (C, D) at concentration OD = 1 were dissolved in saline and Imuregen and plated from dilution to -4 (right side) and -6 (left side) on suitable culture plates.

Table 1. Summary of Imuregen's bactericidal effect.

Bacterium	Ø Saline (S)	Ø Imuregen (I)	I/S × 100	p-value*
<i>Escherichia coli</i> CCM 3954	96	76	79.17	0.4357
<i>Staphylococcus aureus</i> CCM 4223	133	134	100.75	0.9999

*Significance was calculated from Experiment 3 data.

Francisella tularensis strain LVS ATCC 29684, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* CCM3955 were used to verify whether the bactericidal effect of Imuregen on gram negative bacteria was indeed nonsignificant. Although the numbers of bacterial colonies were in all these cases reduced, the reduction of between 5% and 12% was really insignificant (data not shown).

Histology of gut tissue samples

Histological examination of the gut samples of ileum revealed neither pathological changes in morphology of intestinal villi nor significant differences in proportions of enterocytes and goblet cells in histological sections. Evaluation of morphometric data proved differences in heights of intestinal villi between experimental and control group to be statistically insignificant, and there were only insignificant differences in the shape of villi (Fig. 4). All cell types present in wall of ileum (i.e. goblet cells, columnar cells, endocrine cells, and Paneth cells) in the control and experimental samples were the same in the number and the shape. Collectively, the construction of the gut wall and the mucosa with villi showed no morphological differences between control and experimental samples.

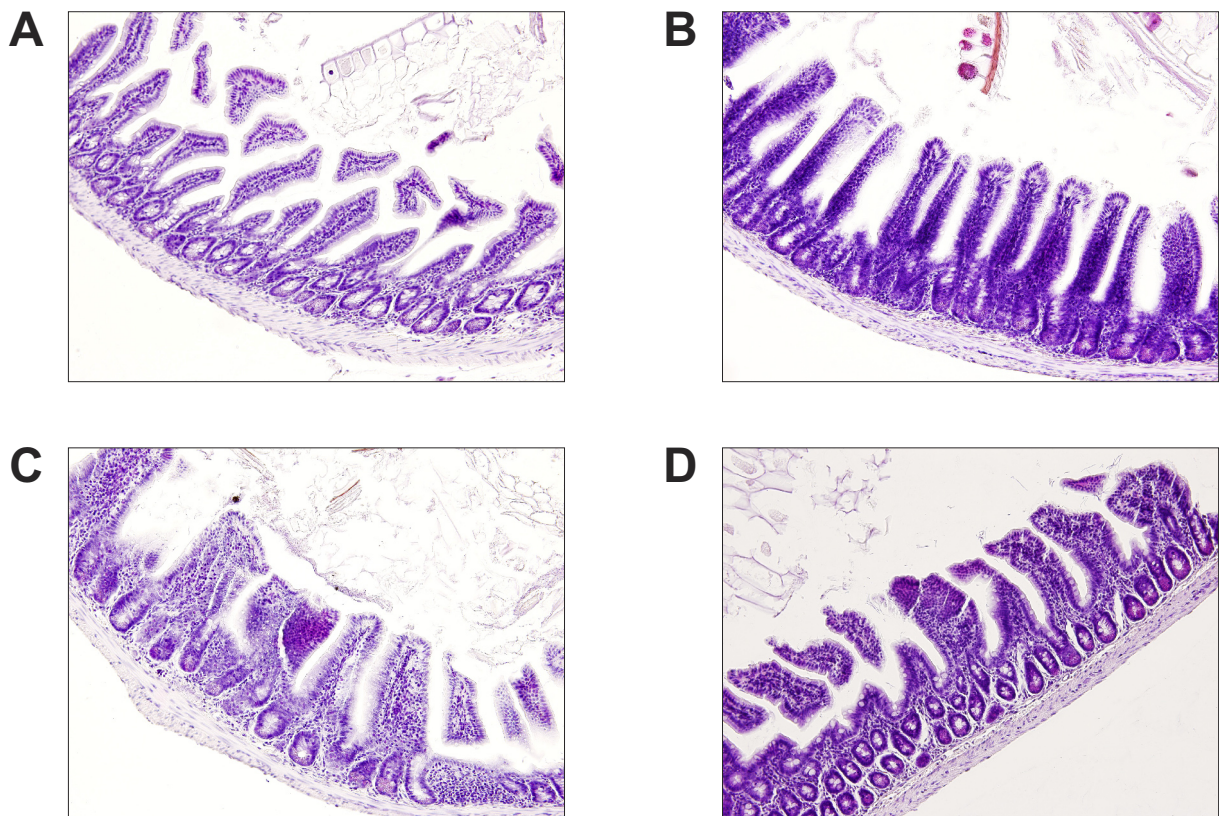


Figure 4. Histological examinations of ileum samples excised from control (A, B) and Imuregen-treated mice (C, D).

Quality of collected samples

In total, 1,660,029 reads were analyzed in this study. Median and mean sequence coverage were 38,056 and 41,500 reads per sample, respectively. Sample coverage ranged from 13,719 reads in the sample with the lowest coverage to 79,315 reads in the sample with the highest coverage.

Imuregen modulates the *Bacteroidetes*/*Firmicutes* ratio

The 33-day treatment of mice by drinking regime with water enriched with Imuregen induced changes in the gut microbiota in favor of phylum *Bacteroidetes* at the expense of *Firmicutes* in comparison to control mice that had only water (Tab. 2, 3). The shift in bacterial phyla was significant only in the colon ($p = 0.0412$ for *Bacteroidetes*; $p = 0.02982$ for *Firmicutes*). Insignificance of phyla shift in the ileum was due to great variability in relative abundance of bacterial phyla within individual mice.

Table 2. Relative ratio of phyla *Bacteroidetes* and *Firmicutes* in ileum of control and Imuregen-treated mice. This ratio was calculated from summary of relative phyla abundance in 10 mice of each group.

	<i>Bacteroidetes</i>	<i>Firmicutes</i>	Bact/Firm ratio
Control	15.9893514	80.1311004	0.199539
Imuregen	27.3754351	69.5114609	0.393826

Table 3. Relative ratio of phyla *Bacteroidetes* and *Firmicutes* in distal colon of control and Imuregen-treated mice. This ratio was calculated from summary of relative phyla abundance in 10 mice of each group.

	<i>Bacteroidetes</i>	<i>Firmicutes</i>	Bact/Firm ratio
Control	29.7164933	64.3976840	0.46145279
Imuregen	40.7785299	53.6281294	0.76039441

Bacterial genera affected by Imuregen administration in murine ileum or cecum

Imuregen treatment significantly affected abundance of 18 genera in the ileum. Twelve genera belonging to phylum *Firmicutes* were more abundant in control mice (i.e., decreased in Imuregen-treated mice). Only 3 genera belonging to phylum *Bacteroidetes* were affected in the ileum and, among these, only the unclassified genus abundance increased in the ileum of Imuregen-treated mice in comparison to controls (Tab. 4).

In the cecum, only 6 genera were of significantly different abundance in control and Imuregen-treated mice. Four genera belonging to phylum *Bacteroidetes* were more abundant in the ceca of Imuregen-treated versus control mice. On the other hand, only a single genus belonging to *Firmicutes* increased in the cecum following Imuregen administration (Tab. 4).

DISCUSSION

Intestinal microbiotas of mice and humans are mixture of different phylogenetic species dominated by the four microbial phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, which comprise 98% of the intestinal microbiota (24–30). Although a basic property of living matter is to create diversity and occupy all available space, microbes are not arbitrarily distributed in the environment (31). Concerning the gut, the spatial localization of microbiota phyla is potentially influenced by mucosa as one of the organizing factors. Distinctive microbial populations have been found near the mucosa of mice as well as of humans (32–36). While *Firmicutes* tend to colonize the mucin layer, *Bacteroidetes* have been shown to be enriched in the luminal content (37,38). Additional details have recently been reported to the original data (39–41). Moreover, a second organizing factor can be binding affinities of strains to different substrates, such as mucus or food particles, and these may be accompanied by varying degrees of replication in a preferred microhabitat (42).

In this research, Imuregen changed the *Bacteroidetes/Firmicutes* ratio in favor of *Bacteroidetes* throughout the entire intestine. In most clinically significant situations, however, the opposite effect on this ratio has been demonstrated repeatedly. For example, obese ob/ob mice were shown to have a 50% reduction in the abundance of *Bacteroidetes* and a proportional increase in *Firmicutes* in comparison with lean mice (43). Similar conclusions have been reached also for obese people (44,45). Reduction in the proportion of *Bacteroidetes* with respect to *Firmicutes* was observed also in patients affected by inflammatory bowel disease in comparison to healthy controls (46–48). A more significant effect on the *Bacteroidetes/Firmicutes* ratio was demonstrated in patients with colon cancer, where the *Firmicutes* genera strongly dominated (47). A similar shift in the *Bacteroidetes/Firmicutes* ratio has been demonstrated also for children suffering with autism spectrum disorder (49) and people under chronic stress (50).

Corresponding to the comparison at phylum level, a comparison at the genus level confirmed that genera belonging to the Gram-positive *Firmicutes* were less abundant both in the ileum and cecum of Imuregen-treated mice. It seems, however, that the major effect of Imuregen was expressed mainly in the ileum, inasmuch as the abundances

Table 4. List of significantly affected genera in ileum or cecum of Imuregen-treated mice.

Phylum	Genus	More in control mice *	More in Imuregen treated mice *
ILEUM			
<i>Actinobacteria</i>	<i>Bifidobacterium</i> #	13.60	
<i>Bacteroidetes</i>	<i>Bacteroides</i>	2.43	
	<i>Prevotella</i>	6.35	
	<i>Other</i>		2.74
<i>Firmicutes</i>	<i>Enterococcus</i>	41.96	
	<i>Clostridium sensu stricto</i>	41.58	
	<i>Anaerostipes</i>	54.04	
	<i>Blautia</i>	38.41	
	<i>Coprococcus</i>	4.66	
	<i>Roseburia</i>	5.84	
	<i>Ruminococcus</i>	3.32	
	<i>Acetanaerobacterium</i>	2.41	
	<i>Faecalibacterium</i>	16.57	
	<i>Gemmiger</i>	18.79	
	<i>Ruminococcus</i>	10.22	
	<i>Megamonas</i>	4.27	
<i>Proteobacteria</i>	<i>Psychrobacter</i>	4.89	
	<i>Other</i>	3.20	
CECUM			
<i>Actinobacteria</i>	unclassified <i>Coriobacteriaceae</i>	1.67	
<i>Firmicutes</i>	unclassified <i>Lachnospiraceae</i>	1.38	
<i>Bacteroidetes</i>	<i>Barnesiella</i>		1.79
	unclassified <i>Porphyromonadaceae</i>		3.75
	<i>Parabacteroides</i>		3.96
	<i>Hallella</i>		3.16

*Relative abundance

Note: *Bifidobacterium* was 13.6-fold more present in the small intestine microflora of control mice compared to the microflora of Imuregen-treated mice. Conversely, non-genus *Bacteroidetes* occurred at 2.74 higher proportions in the small intestine of Imuregen-treated mice. Exactly, the genus *Bifidobacterium* accounted for 0.0418% of the total microflora in the small intestine of control mice, whereas in the Imuregen-treated mice, *Bifidobacteria* accounted for only 0.00308% of the total microflora. The ratio between 0.0418 and 0.00308 is that value of 13.6

of more genera were affected there after its administration. Moreover, it appeared that Imuregen in the ileum mainly suppressed growth of genera from the phylum *Firmicutes* rather than supported the growth of genera belonging to *Bacteroidetes*. In the majority of cases, abundance of *Firmicutes* genera significantly decreased in Imuregen-treated mice, and that could create the appearance of increase in *Bacteroidetes*. Imuregen's effect was slightly weaker in the cecum, likely due to the fact that a majority of its components already had been absorbed by the host within the small intestine. In accordance with the data presented here, the modulatory effect of Imuregen on the gut microbiota seems to result from the host's internal modulation of gut physiology and/or immunology rather than due to the quite insignificant bactericidal activity of Imuregen.

Targeted modulation of microbiota composition thus appears to be a suitable intervention strategy in relation to specific health problems. The so-called Soylent 2.0 diet provides an example demonstrating that such modulation is possible. The Soylent 2.0 diet is a liquid formulation consisting of primarily soy proteins, algal oil, and isomaltulose, as well as smaller amounts of other ingredients, such as essential vitamins and minerals (51). Among Soylent dieters a significant increase in the ratio of *Bacteroidetes* to *Firmicutes* abundance has been found. Whether targeted shifts in the microbiota composition affect the health status of patients with specific diagnoses is presently unknown.

In summary, the data collected from the experiments with per oral application of Imuregen using the drinking regime demonstrate the possibility for Imuregen to modulate the *Bacteroidetes/Firmicutes* ratio in favor of *Bacteroidetes* genera. Moreover, it was shown that the utilization of Imuregen causes no pathological changes in intestinal epithelium. These functional characteristics together suggest that application of Imuregen can be promising in a clinical situation when, due to dysbiosis, the *Bacteroidetes/Firmicutes* ratio has been changed in favor of the *Firmicutes* genera.

Author contribution

Klara Kubelkova: Conceptualization, Investigation, Methodology, Data curation, Writing original draft.
Ivan Rychlik: Investigation, Methodology, Data curation, Writing original draft
Magdalena Crhanova: Methodology, Investigation
Daniela Karasova: Methodology, Investigation
Dasa Slizova: Methodology, Investigation
Jitka Zakova: Methodology
Lenka Luksikova: Methodology
Ales Macela: Conceptualization, Investigation, Methodology, Data curation, Writing original draft.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

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Adherence to Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

Conflict of Interest

The authors declare that they have no conflicts of interest regarding the publication of this article.

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