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High-throughput workflow for cultivation and characterization of gut microbiota strains with anti-inflammatory properties and metabolite signature associated with gut-brain communication

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The gut microbiota is deeply interconnected with the brain, a phenomenon often referred to as the gut-brain axis. Dysfunction in the microbiota-gut-brain axis can cause various neurological and psychiatric disorders associated with chronic inflammation and gut microbiota dysbiosis. Therefore, cultivation of anaerobic human gut microbiota strains, and characterization of their safety status and immunomodulatory potential could contribute to deciphering the molecular mechanisms underlying the microbiota-gut-brain communication and revealed their biotherapeutic potential. However, poor cultivability of gut microbiota members, makes research into their physiological role challenging. Hence, we report a high-throughput workflow based on targeted cultivation linked to metagenome sequencing, combined with the bioinformatic search for gut members with anti-inflammatory properties which produce the most important microbial metabolites that affect brain function. With this approach, we isolated 147 bacterial strains, and 41 were characterized for their immunomodulatory status with 12 strains showing immunosuppressive features with ability of producing brain important metabolites. Through this workflow we established the best growing conditions essential for cultivation, archiving, phenotyping, and characterization of anaerobic gut bacteria important for microbiota-gut-brain-axis research, and characterized the safety and probiotic potential of 7 extremely oxygen-sensitive strains.

Keywords Human gut microbiota, Cultivation, Extremely oxygen-sensitive strains, Immunomodulation, Microbiota-gut-brain axis

Human gut microbiota (HGM) composed of trillions of microorganisms residing inside the human intestinal tract emerged as an important regulator of brain function through complex bidirectional communication often referred to as microbiota-gut-brain-axis (MGBA)[1]. Recent studies have revealed significant changes in the composition and diversity of gut microbiota, i.e. gut microbiota dysbiosis, in various neurological and psychiatric diseases, pointing to their involvement in proper neural function[2–4]. The change in gut microbiota diversity mainly results in the alteration of the composition and concentration of microbial-derived metabolites (MDMs), leading to chronic gut inflammation, which all could influence brain function via direct or indirect routes. Chronic gut inflammation and gut microbiota disturbances have been associated with an epidemic proportion of neurodegenerative and psychiatric diseases, with over 55 million people globally living with Alzheimer's Disease, as the most prevalent neurodegenerative disease, and 280 million people worldwide suffering from

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major depressive disorder, as the most prevalent psychiatric disease[5]. These diseases represent a huge burden on society, impairing the health and the quality of life of affected people and their families.

The complex bidirectional communication between MDMs and the central nervous system (CNS) involves multiple biological networks, including metabolic pathways, neural networks, and the immune system. These networks facilitate the transport of signals generated by MDMs to immune cells in the periphery and the brain. In response, signals are sent back from the brain to the gut wall and the mucosal immune system via the vagus nerve, which enhances gut wall integrity, reduces peripheral inflammation, and decreases the production of pro-inflammatory cytokines. The most important microbial brain messengers described so far are short-chain fatty acids (SCFA) like acetate, propionate and butyrate, important for maintaining integrity of the blood-brain barrier, making it less permeable to harmful substances and regulating neuroinflammation. Butyrate, for example, has been shown to have anti-inflammatory and neuroprotective effects by inhibiting histone deacetylases (HDACs) and modulating microglial activation[6], while propionate and acetate influence neurotransmitter synthesis and immune regulation[7]. In addition, SCFA deficiency has been linked to neurodegenerative and neuropsychiatric disorders, including multiple sclerosis, Parkinson's disease, and depression, in which SCFA deficiency contributes to increased neuroinflammation and impaired brain homeostasis[8]. Similarly, gamma-amino butyric acid (GABA), a major inhibitory neurotransmitter in the brain, plays a key role in reducing anxiety and promoting calmness. A systemic decline of SCFA and GABA have been linked to numerous neurological diseases suggesting the importance of these metabolites in the controlling of neuroinflammation and brain homeostasis[9,10].

Since there is still a large knowledge gap between the frequently detected associations between HGM dysbiosis and neuropsychiatric diseases and the limited number of cultivated MGBA-related strains, the development of high-throughput and integrated methods is of the utmost importance to expand the collections of cultivable HGM members and to facilitate the elucidation of the mechanisms underlying MGBA-related diseases.

Here, we present the original workflow for successful recovery and isolation, cultivation, and characterization of anaerobic gut strains isolated from the feces of healthy human volunteers and for determining their possible association with MGBA, safety status, immunomodulatory effects, and potential probiotic features.

Results

Prediction of HGM metabolic potential for MGBA and bacterial diversity among samples

To evaluate the potential of gut microbiota members of seven healthy donors to produce metabolites important for the gut-brain axis, SCFA and GABA, as well as to determine the taxa with the ability for their synthesis, the shotgun metagenomic sequences of the DNA isolated from fecal samples were analyzed. First, we analyzed the abundance of pathways originating in different taxa and showed that 22 out of 39 known pathways of SCFA production were present in at least some of the samples (Fig. 1A). The analysis of pathways involved in the production of SCFA mixture (Fig. 1B) showed the highest presence of FERMENTATION-PWY (mixed acid fermentation) producing succinate, formate and acetate from glucose in given anaerobic environment of gut lumen, but only three species well-known for SCFA production, were identified, *Faecalibacterium prausnitzii*, *Succinatimonas hippei* and *Escherichia coli* to possess this pathway. Also, the pathway PWY-7383 (anaerobic energy metabolism (invertebrates, cytosol)) and PWY-6590 (superpathway of *Clostridium acetobutylicum* acidogenic fermentation), were highly present in bacteria but also in fungi pointing to the important contribution of these microorganisms to the SCFA production. P162-PWY (L-glutamate degradation V (via hydroxyglutarate)) and P163-PWY (L-lysine fermentation to acetate and butanoate) were not detected in any of the samples. When we analyzed the presence of pathways involved in the production of individual acids, we showed that five out of six known pathways of microbial butyrate production were detected in the majority of samples (only the pathway GLUDEG-II-PWY: L-glutamate degradation VII (to butanoate) was not detected in any of the samples)[11] (Fig. 1C). Pathway PWY-5676 (acetyl-CoA fermentation to butanoate II) was the most abounded in all samples, and the performed analysis identified *Anaerostipes hadrus*, *Coprococcus catus*, *Flavonifractor plautii*, *Intestinimonas butyriciproducens* to use this pathway for butyrate production. Further, CENTFERM-PWY (pyruvate fermentation to butanoate) and PWY-5022 (4-aminobutanoate degradation V) were less common but present in all samples. The other two pathways were 10-1000 times less present, while PWY-5677 (succinate fermentation to butanoate) was present in five out of seven samples, and PWY-8190 (L-glutamate degradation XI (reductive Stickland reaction)) was present only in samples 3 and 6. Except for the PWY-5676, all other pathways of butyrate production were identified only in unclassified sequences. Further, all five pathways known to be involved in propionate production by microorganisms were present in some of the samples (Fig. 1D), but only PWY-7013 ((S)-propane-1,2-diol degradation) was present in all samples with the highest relative abundances. This pathway was identified in *Eubacterium hallii*, *Flavonifractor plautii*, *Intestinimonas butyriciproducens* and *Clostridium perfringens*. P108-PWY (pyruvate fermentation to propanoate I) was detected in all samples except the Sample 6, while PWY-5088 (L-glutamate degradation VIII (to propanoate)) was detected only in Sample 5, and for these as for the other two pathways (PWY-5494 (pyruvate fermentation to propanoate II (acrylate pathway)), PWY-8188 (L-alanine degradation VI (reductive Stickland reaction))) analysis did not identify any specific bacterial species. All propionate-producing pathways were identified at some level in an unclassified category. The pathways involved in acetate production were the most abundant and the analysis of acetate-producing pathways (Fig. 2) showed the dominance of PWY-5100 (pyruvate fermentation to acetate and lactate II) and P41-PWY (pyruvate fermentation to acetate and (S)-lactate I), with the high number of species identified. The species with the highest presence of PWY-5100 were *Bifidobacterium adolescentis* and *Collinsella aerofaciens* in Samples 1 and 2, *Ruminococcus torques* in Samples 3, 4, and 5, and *Blautia wexlerae* in Sample 7. The species with the highest presence of P41-PWY were *Bifidobacterium adolescentis* in Sample 1, *Collinsella aerofaciens* in Samples 2 and 7, *Ruminococcus torques* in Samples 3, 4, and 5, *Blautia obeum* in Sample 6. As expected, the dominant producers of acetate were from the genus *Bifidobacterium*. The GABA production pathways were

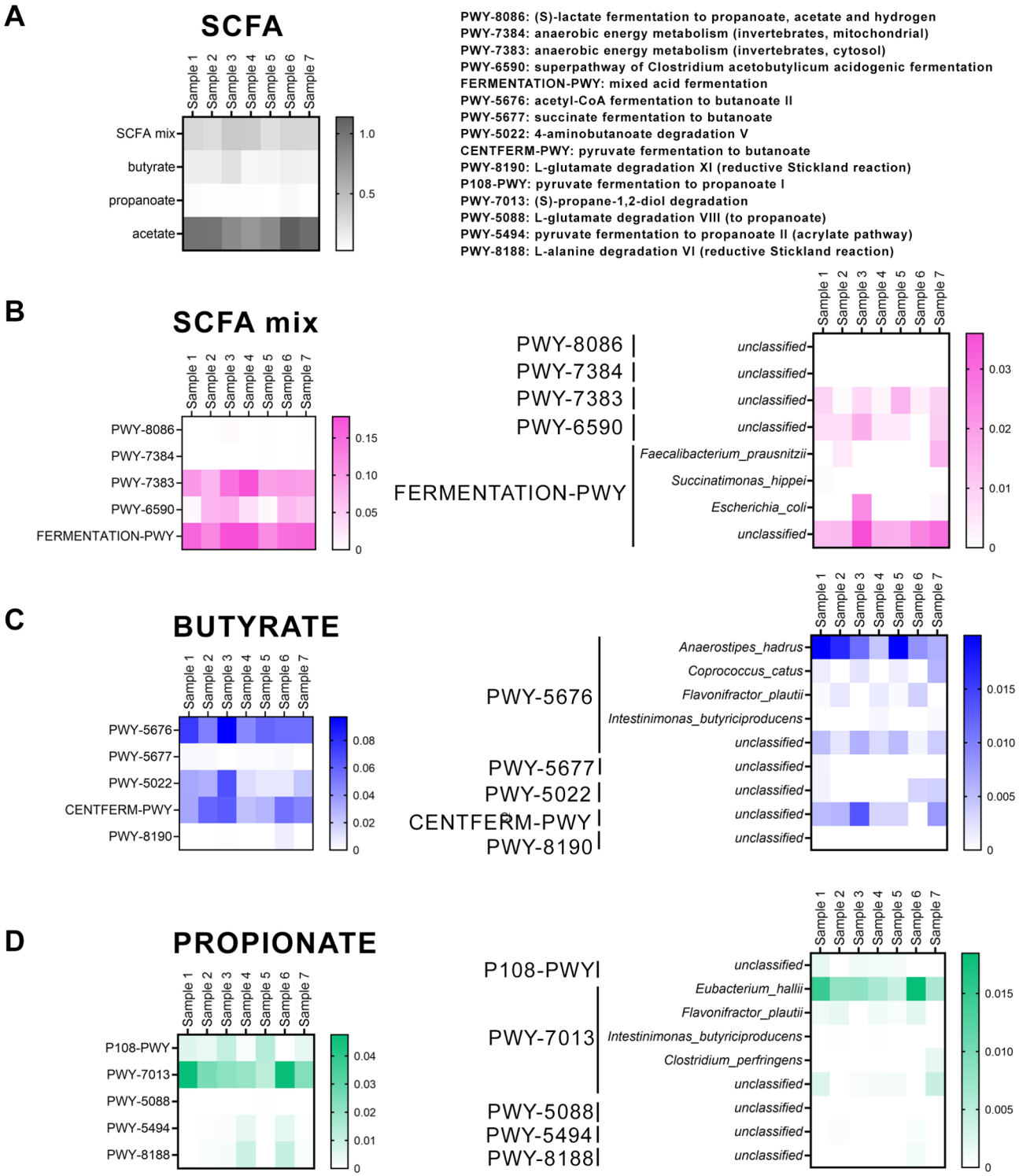

Fig. 1. Heatmaps showing the relative abundances of all pathways (**A**) and individual pathways involved in SCFA mix (**B**), butyrate (**C**), propionate (**D**) production and bacterial taxa containing these pathways, in seven fecal samples used for the following bacteria isolation, based on the shotgun sequencing of the samples.

identified in both *E. coli* and unclassified species, indicating that multiple taxa contribute to GABA synthesis (Fig. 3).

Establishment of a library of HGM bacteria

Further metagenomics analysis (LEfSe, data not shown) showed no significant differences in the composition of fecal microbiota between the samples collected from different donors. Bearing this in mind, as well as our aim

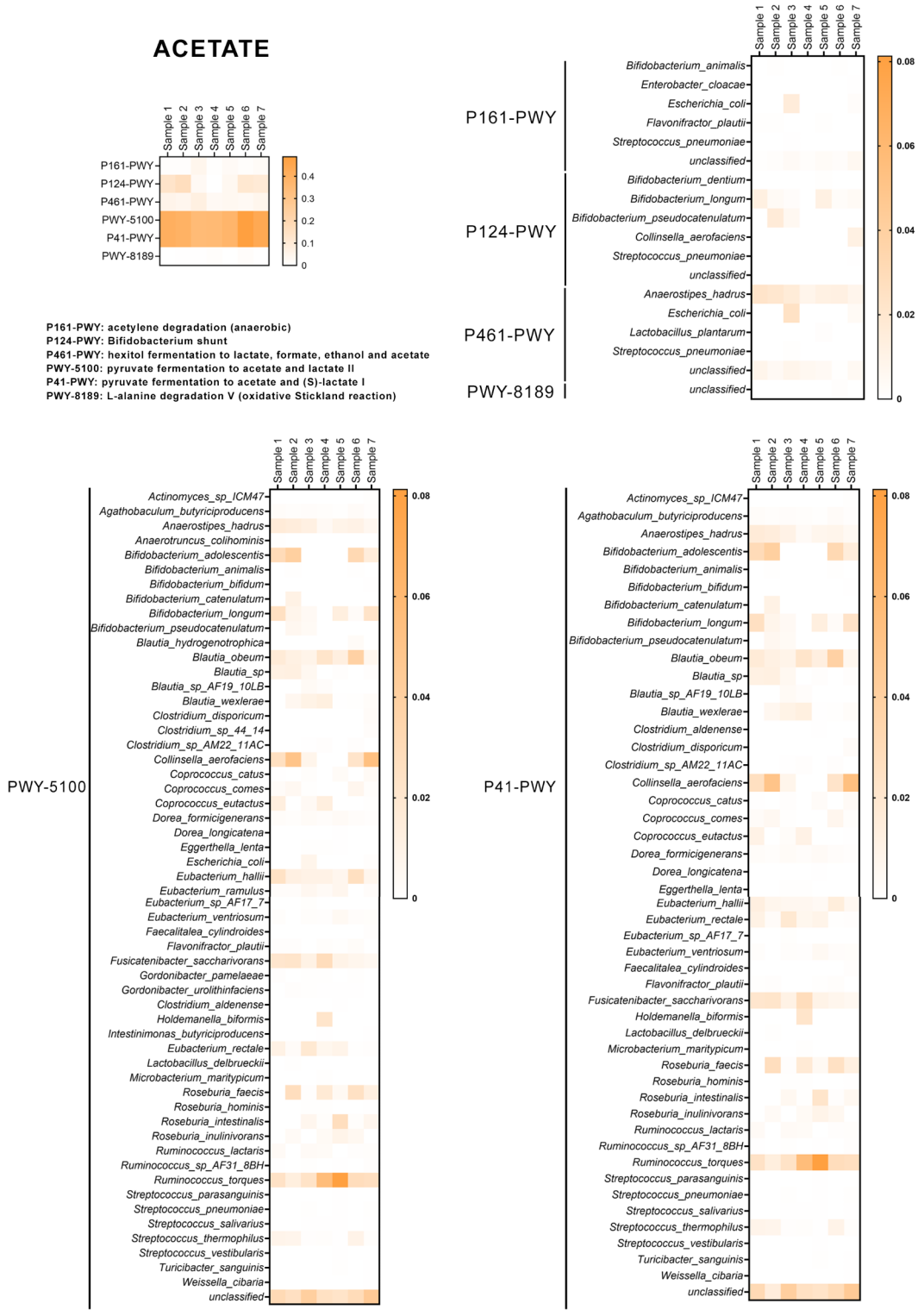

Fig. 2. Heatmaps showing the relative abundances of individual pathways involved in acetate production and bacterial taxa containing them in seven fecal samples used for the following bacteria isolation, based on the shotgun sequencing of the samples.

to isolate as many different strains with the MDMs producing potential, we decided to cultivate each sample in different specific cultivation media. Based on data obtained by metagenomic analysis, the presence of the pathways for synthesis of MGBA-related MDMs (SCFAs and GABA) was confirmed in the seven fecal samples of healthy volunteers, and in addition to some classified producers (predominantly for acetate production), all pathways were detected in unclassified sequences. We considered that yet uncultured bacteria could contribute

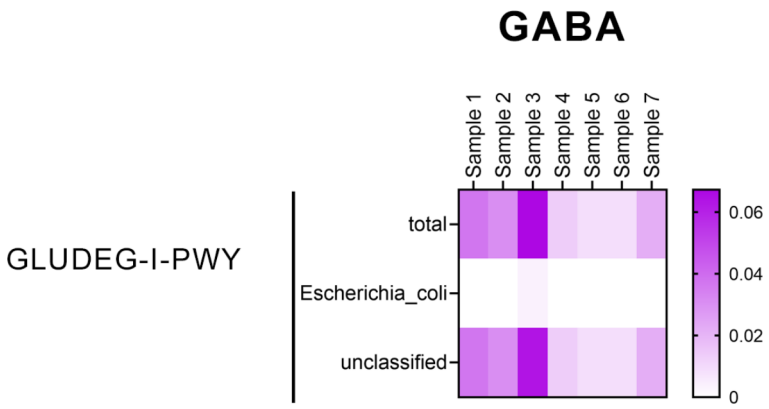

Fig. 3. Heatmap showing the relative abundance of pathway involved in GABA production and bacterial taxa containing it, in seven fecal samples used for the following bacteria isolation, based on the shotgun sequencing of the samples.

to this pool of unclassified sequences, so we decided to pay special attention to the cultivation of strains classified as uncultured based on the 16S rRNA analysis. In addition to uncultured strains, we selected strains classified as species known from literature as potential MGBA-related MDMs producers. Hence, the next step in our workflow was to cultivate selected potential MGBA-related MDMs-producing strains and to further determine their biological activities. The media and cultivation strategies are listed in Supplementary Table S1 and Supplementary Table S2.

In total, 292 colonies were analyzed. Based on the appearance of the colonies and bacterial cells' morphologies, 147 different ones were selected for 16S rRNA sequencing (Supplementary Table S2). The strains that remain cultivable after three subcultivations were stored in 15% glycerol at −80 °C. The proportion of identified extremely oxygen sensitive (EOS) strains by using various methods was between 0 (Sample 1 and Sample 3, mostly bifidobacteria, lactobacilli, and enterococci) and 86.49% (Sample 5), depending on the cultivation conditions. The results revealed that the highest proportion of EOS was achieved in Sample 5, cultivated on CBA, with or without pre-cultivation. In Sample 6, 50% of EOS bacteria were isolated, respectively, together with bifidobacteria. On the other hand, in samples 2, 4, and 7, only sporadic EOS strains were isolated (Sample 2: one strain identified by 16S amplicon sequencing as *Phascolarctobacterium faecium* (non-cultivable) among 55 analyzed colonies, mostly bifidobacteria and lactobacilli; Sample 4: one strain identified as *Bacteroides fragilis* among 36 analyzed colonies, predominantly *E. coli* and *Salmonella*; and Sample 7: one strain identified as uncultured_*Blautia/Ruminococcus* sp. among 39 analyzed colonies, predominantly *Clostridium perfringens*, enterococci, *E. coli* and bifidobacteria).

Molecular phylogenetic analysis of HGM isolates

The 16S rRNA gene sequence clustering successfully segregated 147 HGM isolates into 10 defined subclusters reflecting the biodiversity of the human gut (Fig. 4A). In the constructed phylogenetic tree separation of a subgroup of bifidobacteria (27 isolates), enterobacteriaceae (20 isolates), four subgroups of EOS (43 isolates), *Bacillus* (1 isolate), *Clostridium* (13 isolates), *Enterococcus* (18 isolates) and *Lactobacillus* (25 isolates) could be observed. Subsequently, out of 147 identified strains, 41 strains were selected for further functional characterization based on cultivation requirements, sufficient CFUs necessary for the in vitro host-microbe interaction experiments with the host cells, and the literature data suggesting their beneficial role in various diseases (Fig. 4B). Precisely, 21 EOS strains, 19 bifidobacteria, and 1 *Bacillus* sp. strains were selected for in-depth analysis. To study genetic diversity among selected isolates, we first performed rep-PCR fingerprint analysis with $(GTG)_5$ oligonucleotide to determine potentially similar isolates which could be classified as unique strains. Based on this assay we noticed the similar patterns among some selected strains, mostly bifidobacteria (Supplementary Figure S1). However, without whole genome sequencing of the strains we could not claim that observed similar patterns originated from different colonies of one bacterial strain therefore we included all the isolates in further assays.

Immunomodulatory capability of selected HGM isolates

One of the important features of potential MGBA-related probiotics is their immunomodulatory activity. Hence, the capability of the 41 selected isolates to attenuate gut inflammation was evaluated by testing their potential to suppress the production of IL-8 chemokine in TNF-α/IL-1β stimulated Caco-2 intestinal epithelial cells. The results revealed that 13 out of 41 strains can significantly lower the production of IL-8 by Caco-2 in a pro-inflammatory environment, while 3 strains significantly increase the expression of IL-8 gene (Fig. 5A and B). Out of 13 strains with anti-inflammatory properties, seven strains belong to the EOS, five are bifidobacteria and one *Bacillus* sp. Further, we assessed the cytotoxicity effects of 13 isolates which suppressed IL-8 production in Caco-2 cells to exclude the possibility that the detected reduction of IL-8 is a consequence of cytotoxicity of the strains. Only *Bacillus* sp. NGB259 caused a significant decrease in the viability of the Caco-2 cells and was excluded from further analysis (Supplementary Figure S2), and the work was continued with 12 selected strains.

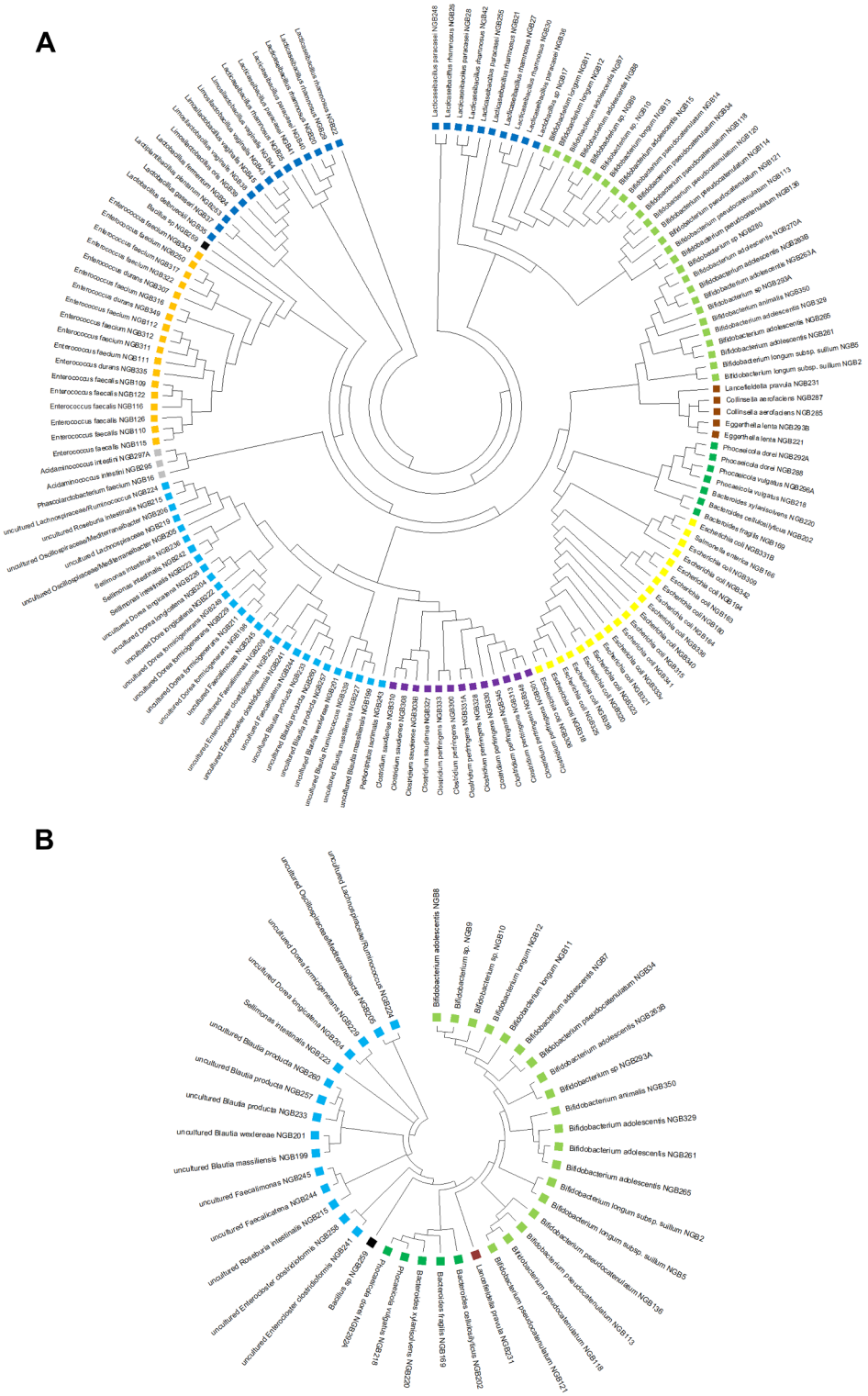

Fig. 4. Maximum likelihood phylogenetic tree constructed based on 147 16S rRNA gene sequences similarities by using MEGA 11 software package showing segregation of HGM isolates into 10 defined subclusters (**A**) and 41 isolates selected for in depth characterization (**B**).

Novel HGM strains synthesize SCFA and GABA in vitro

SCFA and GABA are shown to have immunomodulatory effects in different in vitro and in vivo models, so we further analyzed the capacity of strains with anti-inflammatory potential (12 strains) to produce these metabolites (Fig. 5C) with exact concentrations listed in Supplementary Table S3. Briefly, acetate was detected in 10 isolates, with uncultured_*Blautia producta* NGB233 producing the highest level (21 mM) of this molecule

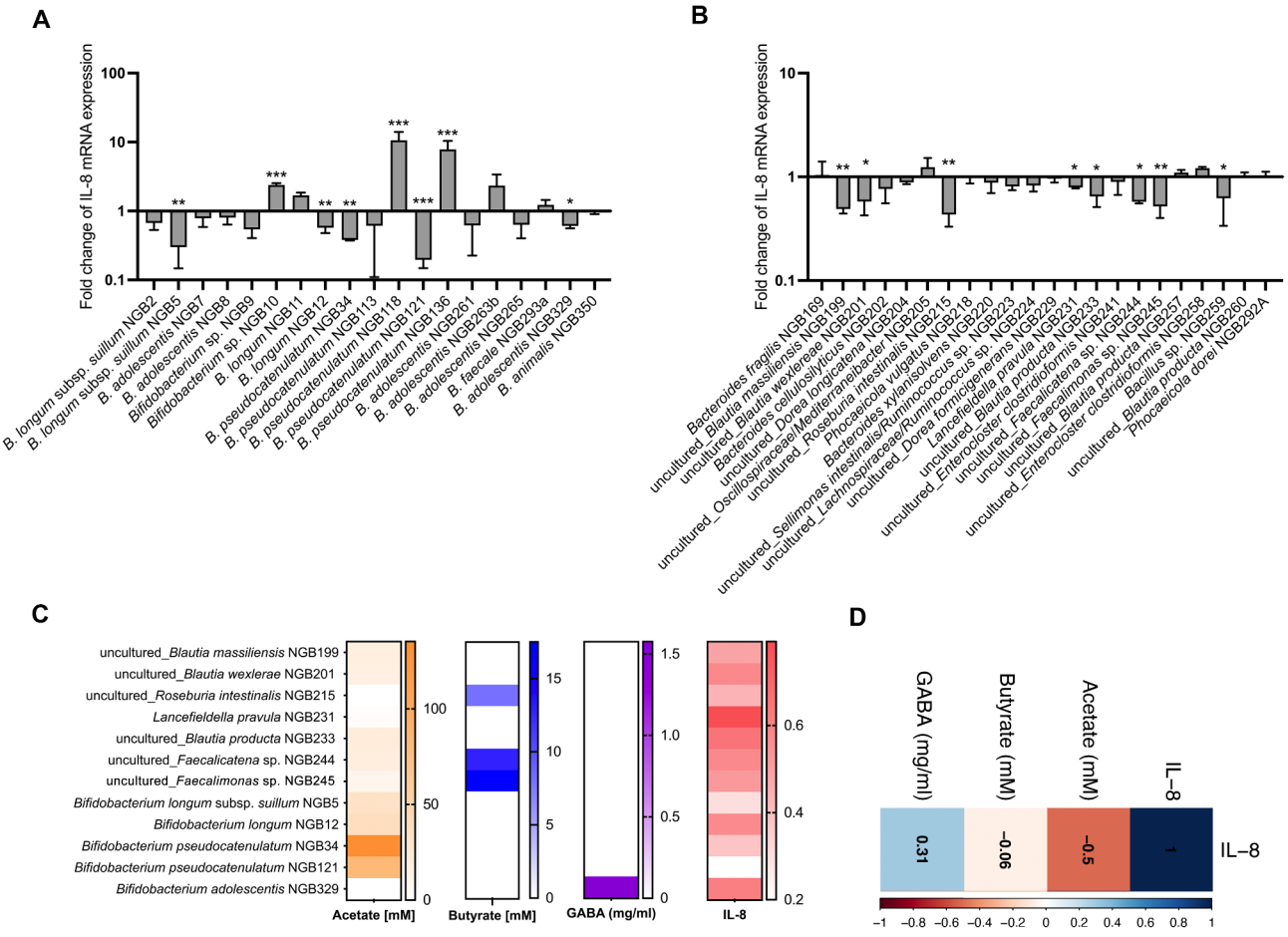

Fig. 5. Capability of IL-8 suppression in TNF-α/IL-1β stimulated Caco-2 cells by selected bifidobacteria (**A**) and EOS HGM isolates (**B**). Relative expression of IL-8 encoding gene was evaluated after 4 h of treatment with 10-fold diluted bacterial culture. The normalized fold change (FC) values of IL-8 mRNA expression were calculated by dividing the FC value of IL-8 mRNA expression in Caco-2 treated with bacterial isolates with the FC value of IL-8 mRNA in Caco-2 cells treated with the corresponding bacterial growth medium. Data are presented as means ± SD. Multiple comparisons between control (bacterial growth medium), inflammation (TNF-α/IL-1β/bacterial growth medium) and bacteria treated cells (TNF-α/IL-1β/10-fold diluted bacterial culture) were done by using one-way ANOVA followed by Tukey's post-hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$ biological replicates). Heatmaps showing the production of acetate (orange) and butyrate (blue) measured by HPLC-UV, and GABA (purple) analyzed by TLC and quantified by ImageJ, in cultures of bacterial isolates shown to inhibit the expression of IL-8 mRNA in Caco-2 cells (red heatmap) (**C**). Spearman's correlation matrix and hierarchical clustering between fold change of IL-8 suppression and SCFA and GABA production by HGM isolates (**D**).

among the EOS isolates and *Bifidobacterium pseudocatenulatum* NGB34 producing the highest concentration of acetate in general (135.2 mM). The production of butyrate was detected only in three HGM isolates; the highest level was produced by uncultured_*Faecalimonas* sp. NGB245 (17.5 mM), followed by uncultured_*Faecalicatena* sp. NGB244 (12.9 mM), both producing butyrate and acetate, and uncultured_*Roseburia intestinalis* NGB215 (8.0 mM) producing only butyrate. The production of propionate was not detected in any of the tested 12 isolates. GABA production was detected in one of the 12 tested strains, *Bifidobacterium adolescentis* NGB329. Interestingly, while acetate was detected in 10 out of 12 tested isolates, including both EOS and aerotolerant species like bifidobacteria, butyrate was detected only in EOS species, while GABA production was detected only in bifidobacteria strain.

Furthermore, although we did not find any statistical significance, the expression of IL-8 mRNA negatively correlated with the ability of the strains to produce acetate and butyrate (Fig. 5D). Although we did not observe a significant correlation between IL-8 suppression and butyrate production, all of the three tested isolates that produce butyrate significantly reduced IL-8. Similarly, while GABA production did not show a significant correlation with IL-8 suppression, the strain that produces GABA significantly decreased IL-8 levels.

Safety assessment of selected HGM isolates

Since 12 strains demonstrated anti-inflammatory potential and no harmful effect on host cells, we further analyzed their susceptibility/resistance to clinically important antibiotics. The antibiotic susceptibility of the 12 selected strains was tested on eight clinically relevant antibiotics including ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol, and the results are presented in Table 1. The four bifidobacteria strains, as well as uncultured_*Blautia massiliensis* NGB199, were found to be susceptible to all tested antibiotics, while *B. pseudocatenulatum* NGB121 showed resistance to streptomycin. All tested isolates were susceptible to vancomycin, tetracycline, and chloramphenicol. Surprisingly, all EOS strains, besides uncultured_*Blautia massiliensis* NGB199, were resistant to at least one antibiotic, mostly streptomycin.

Resistances to specific antimicrobials are indicated in bold. MIC - Minimal inhibitory concentration, Amp - ampicillin, Van - vancomycin, Gen - gentamicin, Str - streptomycin, Ery - erythromycin, Cli - clindamycin, Tet - tetracycline, Chl - chloramphenicol. The values in the table that are bolded indicate the concentration of antibiotics determined to inhibit the growth of the tested strains.

Mucin binding ability of selected isolates

Finally, one of the important probiotic features is their ability to attach to intestinal cells and stimulate surface receptors. Therefore, we performed an in vitro adhesion assay, to test the potential of selected strains to attach to intestinal mucus to increase their resilience in the gut, in order to prolong beneficial effects. The results revealed that 7 strains show the ability to attach to intestinal epithelial stratum to a certain extent with *Lancefieldella pravula* NGB231 as the most potent one, followed by uncultured_*Blautia producta* NGB233 and uncultured_*Faecalimonas* sp. NGB245 (Fig. 6A and B). Altogether, EOS belonging strains showed higher levels of adhesion in comparison to bifidobacteria.

Discussion

The development of high-throughput multi-omics techniques revolutionized microbiota research and led to the creation of an enormous amount of data enabling the characterization of structure, diversity, and dynamics of human gut microbiome[12]. However, the full functional characterization and the understanding of the mechanisms behind the HGM-host cross-talk requires cultivation of gut microbiota members in pure cultures. For this reason, the development and continual optimization of the culturomics approach for the cultivation of novel EOS bacteria emerged as crucial[13].

In this study we introduced the original workflow for the cultivation and functional probiotic characterization of MGBA-related HGM members including: (i) shotgun metagenomics sequencing, searching for the pathways involved in the synthesis of MDMs (SCFA and GABA) related to MGBA; (ii) directed taxa searching for potential MGBA-related MDMs producers; (iii) cultivation of novel MGBA-related MDMs producers; and (iv) characterization of their safety status and probiotic potential (Fig. 7). Using this workflow, a NextGenBiotics (NGB) collection of 147 HGM strains was established, with 41 potentially MGBA-related strains belonging to 25 species of 13 genera (including 21 EOS strains). Among them, 15 strains with the highest percentage matched to the uncultured bacteria, indicating that some of the strains might belong to novel species. Finally, the 12 strains with the anti-inflammatory activity were characterized for their safety and probiotic status.

Although the non-targeted culturomics strategies enable the isolation and cultivation of more diverse microorganisms, they are usually labor-intensive and do not necessarily guarantee that taxa with desired properties will be captured[14]. Targeted strategies can also contribute to broadening the collection of HGM resources for studying their role in particular diseases. One of the major tools for targeting approaches to reach novel bacteria with particular features is metagenomics, providing important information related to their metabolic profile[15].

Alterations in the HGM composition and relative abundance lead to the modification or limitation of synthesis of the crucial bacterial MGBA-related metabolites, resulting in changes in the inflammatory response while depletion of MGBA-related metabolites has been linked to neuroinflammation and a broad range of neuropsychiatric diseases[16]. The levels of SCFAs, the main MDMs produced from indigestible fibers by HGM through the anaerobic fermentation, are changed in neurological and psychiatric diseases, implying that they may be vital to MGBA cross-talk[17]. In particular, butyrate was shown to play a role in neurodevelopment, pointing to the importance of cultivation of butyrate-producing bacteria in pure culture[18]. In addition, the changes in both circulating and brain levels of GABA are associated with HGM changes, playing a role in the modulation of mental health, suggesting that this key neurotransmitter might be a potent mediator of the MGBA[19]. However, the mechanisms behind the role of SCFA and GABA in MGBA cross-talk are still not fully elucidated. Hence, in this research we were interested in isolation and cultivation of novel MDMs-producing HGM members to form a collection of novel strains for studying their role in MGBA. For that reason, shotgun sequencing of DNA isolated from fecal samples of seven healthy human donors was performed, followed by bioinformatic analysis of the abundance of the known pathways for SCFA and GABA production, by using the reference base covering primarily bacteria. The metagenomic analysis applied in this study revealed the presence of the majority of pathways involved in SCFA and GABA production in all samples. Since the unclassified sequences could be from still unclassified and/or uncultured bacteria, in the next step of the selection of isolates identified by 16S rRNA gene sequencing, we decided to cultivate the majority of isolates identified as uncultured based on the NCBI database.

Seeing as the majority of HGM bacteria are considered EOS and are difficult to cultivate, we used various media and cultivation strategies to isolate novel MGBA-related strains from human fecal samples[20]. Interestingly, the highest proportion of EOS was achieved in Sample 5, cultivated on CBA, with or without pre-cultivation, potentially due to the fastidious nature of anaerobic bacteria requiring extremely nutritious substrates or special components in the media, such as CBA[21]. In addition, the pre-incubation in a medium containing reducing

MIC (μg/mL)	Amp	Van	Gen	Str	Ery	Cli	Tet	Chl
B. longum subsp. *suillum* NGB5	1	1	32	64	0.5	0.5	4	2
B. longum NGB12	2	1	32	64	0.5	0.5	8	4
B. pseudocatenulatum NGB34	2	1	64	64	0.5	0.5	8	2
B. pseudocatenulatum NGB121	1	1	128	**256**	2	2	16	8
uncultured_*Blautia massiliensis* NGB199	1	1	16	16	0.5	0.5	4	2
uncultured_*Blautia wexlereae* NGB201	2	1	16	**256**	0.5	0.5	4	4
uncultured_*Roseburia intestinalis* NGB215	1	2	16	**256**	0.5	0.5	4	2
Lancefieldella pravula NGB231	2	2	16	**256**	**4**	**4**	4	8
uncultured_*Blautia producta* NGB233	**4**	4	8	**256**	**4**	**4**	16	8
uncultured_*Faecalicatena* sp. NGB244	**4**	4	32	**256**	0.5	**2**	16	8
uncultured_*Faecalimonas* sp. NGB245	1	1	128	**256**	**4**	0.5	8	2
B. adolescentis NGB329	1	1	32	64	0.5	0.5	4	2

Table 1. Susceptibility (MIC) of selected isolates to relevant antibiotics.

agents cysteine and thioglycolate, was previously shown excellent in anaerobes isolation, pointing to the importance of the reduction potential of the medium. The 16S rRNA gene sequence clustering confirmed that the applied strategy was quite successful since the 147 HGM isolates were grouped into 10 defined subclusters indicating the great diversity of the isolates. Interestingly, as many as 24 strains were annotated as uncultured, according to 16S rRNA gene sequence identity search, sharing the highest identity percent in BLAST algorithm with some already cultivated EOS taxa, pointing to the possibility that these could also be new species. Hence, the establishment of this library of HGM strains represents a notable addition to previous literature data[22,23].

The MGBA cross-talk, through the immune system, keeps the HGM in a balanced state, while if disturbed, might lead to neurodegeneration[24]. Specifically, neuroinflammation is one of the important factors in the pathogenesis of diseases of the CNS. For this reason, the next step in our workflow was the evaluation of the anti-inflammatory activity of the selected 41 isolates. Mucosal immune response, the key player in the epithelial defensive mechanisms, mediated by intestinal epithelial cells secreting a wide array of cytokines, including proinflammatory chemokines, such as IL-8[25]. The relationship between MDMs and the host's inflammatory state has been extensively studied and IL-8 was shown to play a crucial role in that process[26]. The increase in IL-8 production is induced by the presence of other pro-inflammatory factors, such as IL-1β in the case of local inflammation, or lipopolysaccharides in the case of infection[25]. Although this is the basic mechanism of host defense, a similar sequence of events is associated with the development of inflammation described in different neurodegenerative and neuropsychiatric disorders[27]. With this in mind, we evaluated the capability of 41 selected isolates to attenuate gut inflammation by following the production of IL-8 chemokine in TNF-α/IL-1β stimulated Caco-2 intestinal epithelial cells. It was found that the 12 strains (7 belonging to EOS) without the cytotoxic effect on Caco-2 cells, were able to significantly decrease the Caco-2 production of IL-8. The literature data reveal that HGM can decrease inflammation by strengthening the gut epithelial barrier or directly through interaction with components of the immune system that induce inflammation[28]. For example, *Faecalibacterium prausnitzii* can block the activation of NF-κB and secretion of IL-8 in Caco-2 epithelial cells through the secretion of anti-inflammatory MDMs[29]. However, the full spectrum of mechanisms behind this immunomodulatory activity remains unexplored.

The immunomodulatory effects of SCFA and GABA towards attenuation of intestinal inflammation could be the important mechanisms of their neuroprotective actions[10,30]. So, we further analyzed 12 strains with anti-inflammatory capacity for their potential to produce SCFA and GABA. Results obtained in this study revealed that most of the SCFA-producing strains were able to produce acetate, while three strains, belonging to uncultured_*Roseburia intestinalis*, uncultured_*Faecalicatena* sp. and uncultured_*Faecalimonas* sp. (all belonging to families *Lachnospiraceae*, phylum Firmicutes) were able to produce butyrate. The obtained results are in accordance with the literature data, based on the metagenome sequencing, reporting that acetate is widely produced by a number of HGM members, while only specific, phylogenetically diverse bacterial taxa produce butyrate, mainly belonging to *Lachnospiraceae* and *Ruminococcaceae* families in the Firmicutes phylum[31].

Moreover, we noticed a positive correlation between the attenuation of IL-8 production and the production of SCFA in 10 out of 12 strains, including the 3 EOS butyrate-producing strains (uncultured_*Roseburia intestinalis* NGB215, uncultured_*Faecalicatena* sp. NGB244, and uncultured_*Faecalimonas* sp. NGB245) that significantly suppressed the IL-8 production which is in accordance with literature data reporting that SCFA, particularly butyrate, may alter the production of IL-8 by epithelial cells[25]. The correlation between IL-8 attenuation and SCFA concentrations, as well as the fact that bacteria that produce similar concentrations of acetate don't attenuate IL-8 gene expression to the same degree, point to the importance of other MDMs as mechanisms for HGM bacteria to exert their anti-inflammatory properties.

One of the important demands for potential probiotics is to meet safety requirements, hence, the susceptibility to antibiotics and absence of cytotoxic effect on the epithelial cells are necessary properties of probiotic bacteria[32]. In this study, most of the tested strains showed no cytotoxic activity, only the strain *Bacillus* sp. NGB259 was found to influence the decrease in viability of the Caco-2 cells and was excluded from further analysis. The analysis of antimicrobial susceptibility is enormously important due to the fast evolution and spread of antimicrobial resistance. Although the microbiological breakpoints given by EFSA for the Gram positive

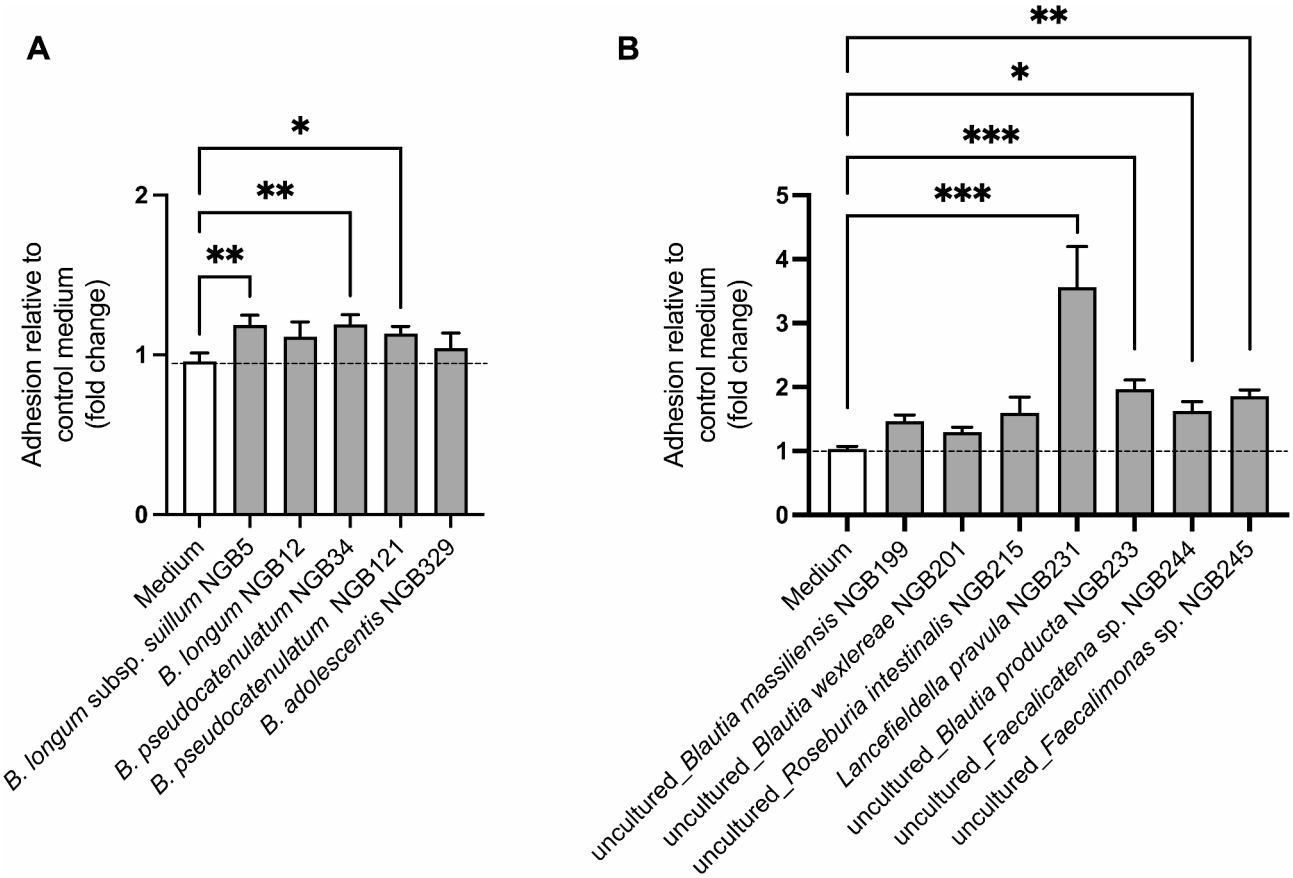

Fig. 6. Mucin binding ability of selected bifidobacteria (**A**) and EOS (**B**) HGM isolates. Percentage (%) of attachment of selected HGM strains to mucin coated plates, in vitro. Data are presented as the means ± SD. One-way ANOVA followed by Tukey's post-hoc test was used to compare the binding percentage of all strains relative to control bacterial culture medium (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 4$ technical replicates, two times repeated experiment).

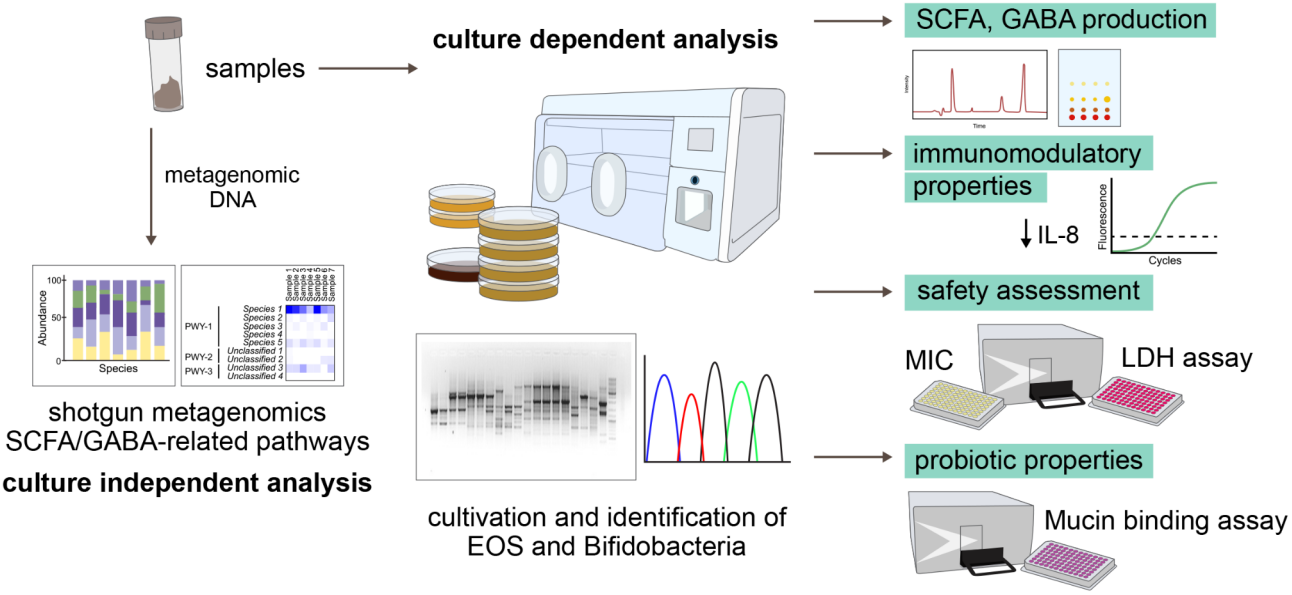

Fig. 7. An overview of the workflow for the cultivation and functional probiotic characterization of gut-brain-axis-related microbiota members including culture independent and culture dependent methods and probiotic characterization analysis.

bacteria are probably not applicable to novel and still uncharacterized taxa, since the information about their natural or acquired resistance pattern is still not available and needs to be revealed, there are some literature data pointing to the resistance of some of them; e.g., more than 50% of the *Fecalibacterium prausnitzii* strains isolated from fecal samples of healthy piglets and calves were resistant to number of antimicrobials comparing to the reference points determined by CLSI for *Bacteroides fragilis* ATCC 25285[33]. Interestingly, our results revealed a high number of the strains (all belonging to EOS group) resistant to various antimicrobials. It is noteworthy that the reference points for these EOS species are still not determined. Since the presence of multiple antimicrobial resistance genes could contribute to the further spread of antimicrobial resistance, further characterization of the strains is needed before regulatory approval.

The ability of mucin binding is an important feature of commensal bacteria enabling their gut colonization[34]. The production of mucus layer by epithelial intestinal cells is one of the key mechanisms by which the host efficiently protects the intestine from a high number of bacteria residing in the gut and keeps intestinal homeostasis. The evaluation of the mucin-binding properties of the selected strains in this study revealed that the highest potential of mucin binding was exhibited by *Lancefieldella pravula* NGB231, uncultured_*Blautia producta* NGB233, and uncultured_*Faecalimonas* sp. NGB245, belonging to EOS strains, pointing to their adaptation for mucus colonization. Since mucin-binding is an important step in the strategy of pathogens to invade the host, the importance of the mucus and their associated commensal bacteria is becoming highly acknowledged in the regulation of infections. However, the exact mechanisms of the interplay among pathogens, commensals and the host are still not fully explored.

In conclusion, the workflow applied in this study enabled the cultivation of 147 novel HGM strains and generated a formidable collection of novel HGM strains. With 21 cultivable EOS strains, 15 strains identified with the highest match as the uncultured bacteria, 12 strains with anti-inflammatory properties reducing IL-8 production, 10 SCFA-producing strains and one GABA-producer, we substantially contributed to the number of potential MGBA-related strains and provided a new source for further investigation of their potential neurobiotic and psychobiotic activity.

Materials and methods

Metagenomic DNA extraction and shotgun sequencing

Metagenomic DNA was extracted from all fecal samples under BSL2 conditions using the Quick-DNA Fecal/Soil Microbe MiniPrep Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions and the Digital Disruptor Genie (Scientific Industries, Bohemia, NY, USA) for samples homogenization. DNA integrity and concentrations were assessed using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan) and a Qubit fluorometer (Thermo Fisher Scientific, USA). Library construction and shotgun sequencing were performed by Novogene (Cambridge, United Kingdom) on the NovaSeq X Plus platform (Illumina, San Diego, CA, USA) in PE150 mode.

Bioinformatics analysis of metagenomics data

Raw data from shotgun metagenomic sequencing were processed using a comprehensive in-house pipeline on the National AI platform (Data Centre, Kragujevac, Serbia) through secure remote access. Initial quality control was performed using FastQC (v0.12.1), followed by trimmomatic, implemented through the KneadData (v0.12.0) environment, to trim adapters and remove low-quality sequences from the Illumina reads. Post-trimming, KneadData utilized Bowtie2 for aligning reads against the human reference genome hg38 (*Homo sapiens* GRCh38) to remove human DNA contamination effectively. The cleaned and high-quality reads were then taxonomically classified using Kraken2 (v2.1.3) with a standard database (RefSeq, version from January 2024). Abundance estimation was provided by Bracken (v2.9) for precise microbial composition analysis. Taxon-specific metabolic profiling was performed with HUMAnN (v3.8) pipeline to obtain pathway abundances and relative abundances of gene families (summarized as CPM normalized level4EC categories). Differences in the microbial composition between the fecal samples collected from different donors were analyzed using the linear discriminant analysis Effect Size (LEfSe) method on the command line[35].

Isolation of novel gut microbiota strains from fecal samples

Fresh fecal samples were collected from seven healthy volunteers (average age 37.8 ± 10.2, four female, three male), and signed informed consent was obtained from all subjects and/or their legal guardian(s). The study was approved by the Ethics Committee of the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia, the approval No. O-EO-019/2020, and all methods were performed in accordance with the Declaration of Helsinki. None of the volunteers had any gastrointestinal tract disorders or had taken antibiotics and probiotics in the prior three months. Samples were collected in sterile tubes immediately after defecation and processed within one hour to ensure sample integrity. At the moment of sampling, it was instantly submerged in the protective medium (approximately 5 g of feces in 50 mL of protective medium, with the container being filled to the top), as previously described[36]. The samples were transferred immediately into a Whitley A35 anaerobic workstation (Bingley, UK) with an atmosphere of 10% H_2, 10% CO_2, and 80% N_2, at 36 °C. A 1 g piece of each fecal sample was resuspended in 10 ml of phosphate-buffered saline (PBS) containing 0.1% L-Cysteine-HCl (Sigma). After thorough vortexing to ensure homogeneity and allow for the suspended fecal particles to settle at the bottom of the tube, the upper parts of the suspension were used for the preparation of serial dilutions (10^{-1} to 10^{-6}) using PBS. The 0.1 mL of dilutions were spread onto the surface of various culture plates, including Gut Microbiota Medium (GMM) agar[37]; Brain Heart Infusion (BHI) agar, Gifu Anaerobic (GAM) agar (HiMedia), Minimal Medium supplemented with Inulin (MMI) agar, Peptone Yeast Glucose (PYG) agar, Yeast Casitone Fatty Acids (YCFA) agar, Columbia Blood Agar (CBA). Compositions of all media used are listed in Supplementary Table S1. All plating procedures were carried out inside the anaerobic chamber. After 2, 7, and

14 days of incubation at 36 °C in strictly anaerobic conditions, colonies were selected for further cultivation. To ensure the isolation of a high diversity of gut bacteria, the modifications in sample preparation, media, and cultivation conditions were introduced, as presented in Supplementary Table S2. In addition to the plating of fecal dilutions, undiluted samples were inoculated (10%) in liquid GMM and MMI and incubated in the same conditions as above (pre-inoculation step) (samples 1 and 2). Sample 4 was cultivated only in GAM medium, with and without supplementation with succinate (13.5 g/L). Sample 5 was spread onto CBA plates. A pre-cultivation step was employed with the goal of increasing the diversity of isolated strains by allowing minority population bacteria to increase in abundance. For this purpose, 50 µL of the initial suspension was added to 10 mL of a pre-cultivation Soybean-Casein Digest (SCD) medium: 27.5 g/L Soybean-Casein Digest, 2 g/L Yeast extract, 0.5 g/L Meat extract, 2 g/L Glucose, 0.005 g/L Hematin, 0.0005 g/L Menadione, 0.2 g/L Sodium Citrate, 1 g/L L-Cysteine, 0.5 g/L Sodium Thioglycolate. After 7 days of pre-cultivation, serial dilutions of the culture were made, and 0.1 mL of each dilution was spread onto CBA plates. Sample 6 was spread onto the YCFA agar and PYG agar plates. Sample 7 was plated on BHI agar, PYG agar, WCA agar, BBA, GAM agar, GAM/blood agar plates. Plates were incubated anaerobically at 36 °C for the next 48–72 h. After incubation, individual colonies with distinct morphologies were selected and subcultured onto fresh agar plates to obtain pure cultures. Purity was ensured by repeated subculturing. Pure bacterial cultures were stored in 15% (v/v) glycerol at − 80 °C for long-term preservation.

Genomic DNA isolation and 16S rRNA gene sequencing

Bacterial DNA was isolated from overnight-grown pure bacterial cultures and DNA quality and concentration were assessed by using BioSpec-nano spectrophotometer (Shimadzu, Japan). The PCR amplification of 16S rDNA was done with UNI16SF (5′-GAGAGTTTGATCCTGGC-3′) and UNI16SR (5′-AGGAGGTGATCCA GCCG-3′) primers described previously[38]. The obtained PCR amplicons were purified by using GeneJet PCR Purification Kit (Thermo Fisher Scientific) and sequenced in Macrogen Service (Amsterdam, Netherlands). The BLAST tool was used to determine the DNA sequences most similar to those in the NCBI GenBank database for the identification of isolated gut bacteria.

Construction of phylogenetic tree

Phylogenetic analysis based on 16S rRNA was conducted in MEGA 11 software package by using the Tamura-Nei model of maximum-likelihood (ML) method[39]. Bootstrapping of 1000 replicates was used to infer the confidence levels of ML trees. The tree with the highest log likelihood (-12776.2819) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value.

Rep-PCR analysis

The bacterial DNA extracted from 24 isolates to evaluate potentially similar strains among HGM isolates were used as a template for PCR-based fingerprint analysis. The repetitive element palindromic-polymerase chain reaction (rep-PCR) analysis with $(GTG)_5$ oligonucleotide (GTGGTGGTGGTGGTG) was performed as previously described[40]. The PCR products were analyzed on 1% agarose gel electrophoresis and visualized by a Biometra CCD camera BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).

Antimicrobial susceptibility testing

For the assessment of the susceptibility to antimicrobials of bacterial strains, a serial two-fold dilution procedure in broth was used. The broth microdilution method and selection of antimicrobials were performed according to EFSA Panel[41] and previosly published works[42,43]. Antimicrobials used were ampicillin (1–4 µg/mL), vancomycin (1–4 µg/mL), gentamicin (8–32 µg/mL or for *Bifidobacteria* sp. 32–128 µg/mL), streptomycin (16–64 µg/mL or for *Bifidobacteria* sp. 64–256 µg/mL), erythromycin (0.5–2 µg/mL), clindamycin (0.5–2 µg/mL), tetracycline (4–16 µg/mL) and chloramphenicol (2–8 µg/mL). Two-fold serial dilutions of each antimicrobial were prepared in Hi-Sensitivity Test Broth (Himedia Laboratories, India) for *Bifidobacteria* sp., or for other tested strains in PYG broth. Testing was performed in triplicates, with 200 µL of antimicrobials dilution or control media poured into 96-well microplates (Sarstedt, Nümbrecht, Germany) and 20 µL of cell concentration set to 0.5 McFarland of selected strains were inoculated in 96 well plates. Microplates were incubated anaerobically at 37 °C for 24–48 h, and the assessment of the susceptibility to antimicrobials was quantified spectrophotometrically at 600 nm using plate reader Tecan Infinite 200 Pro (Tecan Group Ltd. Switzerland). After incubation, the MIC was defined as the lowest antimicrobial concentration that inhibited visible bacterial growth.

GABA detection by Thin-Layer chromatography (TLC)

Selected isolates from the fecal samples of healthy donors were analyzed for GABA production by TLC. Bacteria were grown in a culture medium supplemented with 3 mg/mL of monosodium glutamate, precursor for GABA synthesis, (Acros organics, Morris Plains, NJ, United States) for 48 h. The cells were harvested by centrifugation, and 1 mL of the supernatant was evaporated to a volume of 200 µL, and 400 µL of 7% acetic acid was added. The samples were centrifuged at 8000 × g for 15 min. The resulting supernatants were subjected to TLC analysis. For TLC, 1 µL of the supernatant was spotted onto a DC-Fertigfolien ALUGRAM Xtra SIL G/UV_{254} TLC plate (Macherey-Nagel, Düren, Germany) and developed using a solvent system consisting of n-butanol, acetic acid, and water (4:1:1 v/v/v) with 0.2% ninhydrin reagent added for visualization. Once development was complete, the plate was dried.

HPLC-UV assessment of bacterial production of SCFAs

SCFA (acetate, propionate, and butyrate) concentrations were measured in bacterial cultures. Growth media were supplemented with soluble starch (1 g/L), cellobiose (2 g/L), lactic acid (8 mM), inulin (2 g/L), and acetate (30 mM; for butyrate production) for culturing bifidobacteria (MRS medium) and soluble starch and cellobiose for all other strains (PYG medium), in order to stimulate SCFA production. Bacterial cells were pelleted, and metabolites were quantified in culture supernatants. All HPLC measurements were performed using the UltiMate 3000 UHPLC system (HPLC-UV) (Thermo Scientific, Breda, the Netherlands). The system was operated using the Chromeleon software version 6.8 (Thermo Fisher Scientific, MA, USA). SCFA extraction was performed according to described method[44]. Chromatographic separations were performed on a Hypersil Gold aQ column (150 × 4.6 mm i.d.) with a particle size of 3 µm (Thermo Scientific, Waltham, MA, USA) protected by a guard column. The mobile phase consisted of acetonitrile (Fisher Chemical, Fair Lawn, NJ, USA) and 20 mM NaH_2PO_4 (Serva, Heidelberg, Germany) with pH 2.2. The elution program used is described in Supplementary Table S4. The column compartment temperature was maintained at 30 ℃. The injection volume for each sample was set to 10 µL. Chromatographic separation was monitored at a wavelength of 210 nm. Quantification of SCFAs was done using the external standard calibration method. The concentrations of SCFA are expressed in mM. Solutions of SCFA standards (Sigma-Aldrich, St. Louis, Missouri, USA) were prepared at concentrations of 1 mM, 2.5 mM, 5 mM, 10 mM, and 25 mM. Succinic acid was used as an internal standard to compensate for variations in sample preparation.

Mucin adhesion assay

Adhesion to mucin has been done as previously described[32]. The 96-well plate was coated with mucin type III from porcine stomach (Sigma-Aldrich) in a final concentration of 10 mg/mL. Overnight bacterial suspensions were adjusted to 1.5 McFarland standard units in culture media of each strain and then incubated for 3 h at 37 °C in the anaerobic chamber. After incubation, wells were washed with PBS and attached bacteria stained with 10% crystal violet solution for 30 min. Finally, wells were washed with PBS, and 96% ethanol was added to dissolve the dye. Optical density was measured at 583 nm in Tecan Infinite 200 Pro (Tecan Group Ltd. Switzerland). All the experiments were performed in quadruplicate. The adhesion values have been normalized to the control medium, and the results are presented as fold change values.

Cell culture and treatments

Human Caco-2 cells (ECACC 86010202) obtained from the European Collection of Authenticated Cell Cultures were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) GlutaMAX (GIBCO), containing 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin-streptomycin (GIBCO). The cells were grown at 37 °C in 5% CO_2. At approximately 90% confluence, the monolayers were detached by incubation with a Trypsin solution (Sigma-Aldrich) for 5 min at 37 °C. Then, 2×10^5 cells per well were seeded in 24 well plates, and the medium was replaced every two days. Seventeen days post-confluence Caco-2 monolayers were treated with 10 ng/mL of both TNF-α and IL-1β in order to induce inflammation. Then, cells were immediately treated with 10-fold dilutions of bacterial cultures obtained after 24 h (bifidobacteria) or 48 h (EOS strains) in media supplemented with soluble starch, cellobiose, inulin, lactic acid or acetate and incubated at 37 °C and 5% CO_2. After 4 h, supernatants were collected, and cells were harvested with 0.5 mL Trizol (Invitrogen).

Lactate dehydrogenase (LDH) cytotoxicity assay

Cellular cytotoxicity was assessed by quantifying lactate dehydrogenase (LDH) activity in the supernatants. LDH activity was measured spectrophotometrically by CyQUANT LDH Cytotoxicity Assay Kit (Thermo Fisher), as per the manufacturer's instructions. Briefly, Caco-2 cells were treated with 10 ng/mL of both TNF-α and IL-1β and 10% of bacterial cultures, and LDH activity was determined by measuring the absorbance at 490 nm and 680 nm, using a plate reader Tecan Infinite 200 Pro (Tecan Group Ltd. Switzerland).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Caco-2 cells by using Trizol reagent (Thermo Fisher Scientific). Isolated RNA was treated with DNase I (RapidOut DNA Removal Kit) according to the manufacturer's instructions (Thermo Fisher Scientific) in order to remove potential contamination with genomic DNA. RevertAid Reverse Transcriptase (Thermo Fisher Scientific), random hexamers (Thermo Fisher Scientific), and RiboLock RNase inhibitor (Thermo Fisher Scientific) were used for the transcription of 0.5 µg of isolated RNA. Quantitative real-time PCR (qPCR) was performed under the following conditions: 2 min at 95 °C activation, 40 cycles of 5 s at 95 °C and 30 s at 60 °C in Line-Gene 9600 Plus Real-Time PCR (Hangzhou Bioer Technology) by using IC Green qPCR Universal Kit (NIPPON Genetics, Düren, Germany). Primers specific for IL-8[45] (forward 5′-ACAC AGAGCTGCAGAAATCAGG-3′ and reverse 5′-GGCACAAACTTTCAGAGACAG-3′) were used in the qPCR reaction. GAPDH[46] (forward 5′-GTGAAGGTCGGAGTCAACG-3′ and reverse 5′-TGAGGTCAATGAAGGG GTC-3′) was used as a reference gene for normalization. All results are expressed as relative target abundance by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were expressed as means ± SD. The effects of 10-fold diluted overnight bacterial cultures on Caco-2 cell lines were compared to control and inflammatory conditions by using one-way ANOVA. Post-hoc analysis was performed using Tukey's test to identify significant differences between groups. A p-value less than 0.05 was considered statistically significant. These data were statistically analyzed and visualized using GraphPad Prism software (GraphPad Prism Inc., San Diego, CA, USA). Spearman's correlation matrix was generated to determine the association between fold change of IL-8 suppression and SCFA and GABA production by isolates

using *rcorr* (v5.1-3) and *corrplot* (v092) functions in RStudio (v2024.04.2). The heatmaps showing the relative abundances of SCFA and GABA pathways were made in GraphPad Prism software.

Data availability

All data of this work are presented in this article and in Supplementary material and can also be made available through the corresponding author upon request. Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession code PRJEB79246 (secondary accession number ERP163433).

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Author contributions

Conceived and designed the experiments: NG, JĐ, MD, SSB; intellectual contribution: NG, JĐ, MD, SSB; data acquisition: JĐ, MD, SSB, AB, HM, SJ, DR, EB, JL, MŽ, MT, ATV; data analysis, statistical analysis, and interpretation: JĐ, MD, SSB, AB, HM, SJ, DR, EB, JL, MŽ, MT, ATV; wrote the paper: NG, JĐ, MD, SSB; edited and revised the manuscript: NG, JĐ, MD, SSB.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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