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Research Article

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Abstract

Background

Nutritional interventions have been considered an alternative rationale for preventing Inflammatory Bowel Diseases. The investigation of their impact on the gut microbiota is a fundamental question since shifts in the composition of the commensal bacterial community are required for the onset and maintenance of colitis. Therefore, in this study, we investigated the effects of two types of cheeses, fermented by probiotic propionibacteria. One experimental single-strain cheese was solely fermented by *Propionibacterium freudenreichii* CIRM-BIA129. Another was an Emmental made in industrial conditions using the same propionic strain, in combination with *Lactobacillus delbrueckii* CNRZ327 and *Streptococcus thermophilus* LMD-9, as starters. Both kinds of cheese were tested in healthy conditions and the context of DSS-induced colitis in conventional C57BL6 mice and the gut microbiota was investigated.

Results

Based on the metagenomic analysis, our results suggest that cheese consumption, whatever the kind of cheese, did not disturb the typical microbial community, although the Emmental intake increased symbionts population as *Romboutsia sp.* and partially increased *Akkermansia muciniphila*. Furthermore, metabolic pathway reconstruction analysis suggests that *A. muciniphila* may produce acetate through sulfate assimilatory metabolism and may cooperate with other commensal species in tryptophan and glutamate metabolism to produce indole and gamma-aminobutyric acid. Considering the colitis context, the consumption of the Single-strain cheese restored some of the impaired microbiome metabolic functions, while Emmental cheese promoted the increase of *Ligilactobacillus murinus.* This bacterium presented several genes involved in producing metabolites and adhesin proteins with potential immunomodulatory activity.

Conclusions

This study concludes that the Emmental cheese effects increased the microbiota's capacity to produce metabolites involved in gut-brain axis regulation in intestinal homeostasis condition and, therefore, may represent a potential therapy for inflammatory neurodegenerative diseases. Regarding the colitis context, our results support the beneficial properties of functional Emmental cheese by suggesting possible anti-inflammatory mechanisms based on the promotion of *Lg. murinus* interaction with the host.

Background

Western lifestyle, including diet, plays an important role in the growing prevalence of Inflammatory Bowel Disease (IBD), a set of chronic diseases for which no cure exists. Conventional IBD treatment promotes

strong side effects, responsible for a negative impact on patients' well-being [1]. Therefore, nutritional interventions such as the use of functional foods have been glimpsed as a way to alleviate IBD symptoms [2]. Dairy products, including beverages and cheeses, are interesting candidates for developing novel rationales for treating gut inflammation, as they contain antioxidation and anti-inflammatory bioactive molecules such as vitamins, minerals, fatty acids, and peptides produced by the bacterial fermentation process [3].

Moreover, using probiotic bacterial strains in food may provide additional protective properties. In this context, two Swiss-type experimental cheeses were developed by our research group in an attempt to evaluate their protective capacity in a murine colitis model induced by Dextran Sodium Sulphate (DSS) [4]; One using the probiotic *Propionibacterium freudenreichii* CIRM-BIA129 strain as a unique starter (Single-strain cheese); and a second cheese (Emmental-type cheese) based on the use of three strains, *P. freudenreichii* CIRM-BIA129 and two Lactic Acid Bacteria (LAB), *Lactobacillus delbrueckii* CNRZ327 and *Streptococcus thermophilus* LMD-9. These three strains were selected based on their ability to protect the integrity of the intestinal mucosal barrier, even when challenged by acute inflammation [5]. Although this approach has been demonstrated to ameliorate symptoms of inflammation, the impact of the experimental cheeses on the gut microbiota remains veiled.

Indeed, the composition of the diet is known to modulate the gut microbiome rapidly and reproducibly [6]. Accordingly, dairy products may modulate gut microbiota and immune response [7]. In addition to high amounts of bacteria involved in ripening, cheese may contain prebiotic factors leading to a modification of the composition of the bacterial community in the gut, which could be either beneficial or detrimental to the host's health. Recent studies suggest our microbiome can dynamically respond to environmental changes, such as diet, via a restructuration of microbial species populations, either by selecting adaptation mechanisms or driven by horizontal gene exchange from allochthonous food-borne bacteria [8]. In addition, it is well established that substantial changes in the microbiota are involved in the pathogenesis of IBD and metabolic syndromes such as obesity [9]. On the other hand, the use of Functional foods with live microorganisms has been shown to improve symbiotic functions by increasing the microbiota metabolic processes involved in the production of short-chain fatty acids (SCFA) and other beneficial metabolites with protective roles in the gut and may also affect in a positive way systemic physiology [2], [10].

Given interesting results on the restoration of colitis in mice with the cheese, including down-regulation of the proinflammatory markers Tumor Necrosis Factor (TNF) and Interferon gamma (IFNγ), in the present study, we aimed to investigate the modulatory effects of the experimental cheeses towards the gut microbiota, in homeostasis condition and during inflammation induced by DSS, in the C57BL6 mice model, using high standard Shotgun metagenomic sequencing approach.

Results

Effects of cheese consumption on microbiome structure and function in homeostasis:

Treatment with both kinds of cheese does not disturb microbiome diversity, but Emmental increases the population of Romboutsia **and** Akkermansia **in healthy conditions**

Shannon index analysis revealed no difference (p = 0,686) among the groups of healthy mice, as shown in Fig. 1A, suggesting that the treatment with the two kinds of cheese does not affect the gut microbiota diversity. Regarding the analysis of the relative abundance of taxa (Fig. 1B and Table S1), the five most abundant genera in the control group (Naïve) were *Bacteroides* (64,3%), *Parabacteroides* (8,0%), *Alistipes* (5,5%), *Akkermansia* (3,2%) and *Bifidobacterium* (2,8%). None of those genera populations presented significant changes after consumption of the cheeses. However, *Romboutsia* showed a significant increase in mice consuming the Emmental cheese (0,2%), when compared to the Naïve group (0,02%) (p = 0,029). Although *Akkermansia* did not show a statistically significant difference, it was found enriched (20–60%) in three samples (n = 6) of the Emmental Cheese feeding group.

Treatment with cheeses modulates metabolic and cellular processes involved in microbiota adaptation to environmental stress, sulfur assimilation, and xenobiotics in healthy conditions

The principal component analysis (PCA) of the number of genes in each KEGG pathway category indicates high data homogeneity among healthy mice groups, as shown in Fig. 2A. The analysis of similarities (ANOSIM) indicated no significant difference among the groups (Figure S1) (P = 0,1824). Regarding the functional profiling comparison among the groups (Fig. 2B, C, D, and E and Table S2), the cheese matrix consumption promoted an enrichment of genes involved in the sulfur relay system, RNA polymerase functions, and PI3K-Akt signalling pathway. At the same time, a reduction was observed in neomycin, kanamycin, and gentamicin biosynthesis when compared to the Naïve group. Mice fed on the Single-strain cheese exhibited a decrease in lipopolysaccharide biosynthesis (LPS) and an increase in fatty acids biosynthesis. After treatment with Emmental-type cheese, mice microbiota mainly presented a decrease in gene richness associated with amino sugar and nucleotide sugar metabolism, and sulfur metabolism. However, an increase in benzoate degradation and two kinase signalling pathways was also observed when compared to the Naïve Group.

Effects Of Cheese Consumption On Microbiome Structure And Function In Mice With Colitis:

Microbiota of mice fed with single-strain cheese increases in Proteobacteria during inflammation

The beta diversity analysis indicated no significant difference among the groups (p = 0,0867) (Figure S2).

The genera *Bacteroides* (p = 0,0011) and *Parabacteroides* (p = 5,21E-3) were significantly reduced after DSS administration when compared to the Naïve group (Fig. 3 and Table S3). No statistical difference was found regarding the relative abundance of taxa of mice with DSS-induced colitis treated or not with the cheese matrix (p > 0,05). However, mice consuming the single-strain cheese exhibited an increase in the population of *Helicobacter* (p = 0,016), *Campylobacter* (p = 8,72E-3), and *Arcobacter* (p = 0,026) (Fig. 3 and Table S3).

Microbiota of mice fed with Emmental-type cheese increases in Lactobacillus **during colitis**

Lactobacillus exhibited the most increased population in mice treated with the Emmental-type cheese, followed by *Lacnhoscostridium*, *Bifidobacterium, Blautia*, and *Roseburia* when compared to the DSS Group (p < 0,05) (Fig. 4, Figure S3 and Table S4). The bacterial genera *Bacteroides*, *Prevotella*, *Curtobacterium*, *Alistipes*, and *Pseudomonas* (p < 0,05) were among the taxa with a reduced population in this group.

Consumption of Single-strain cheese during colitis can restore some of the impaired gut microbiota metabolic functions

The PCA of the number of genes per KEGG pathway followed by ANOSIM has shown significant differences among the DSS Group and the other groups (Fig. 5A and Figure S1) (P = 0,0039). Regarding the differentially enriched KEGG pathways among the groups (Fig. 4, Fig. 5B, C, D, and E and Table S5), the DSS administration caused substantial changes, mainly a reduction in the global metabolic processes (Metabolic pathways and Microbial metabolism in diverse environments) followed by specific metabolic processes such as carbohydrate metabolism (Pentose phosphate pathway, Galactose, and Inositol phosphate metabolism), amino acids metabolism (Arginine, Proline, Tryptophan, and Lysine), Lipid metabolism (Glycerolipid, Sphingolipid and Arachidonic acid), Metabolism of co-factors and vitamins (Porphyrin and Vitamin B6), energy metabolism (Sulfur) and xenobiotics metabolism (Nitrotoluene and Styrene degradation) (Fig. 5B and Table S5). However, enrichment was observed regarding other pathways including lipid (Ether lipid and linoleic acid) and amino acid metabolism (Valine, leucine, and isoleucine biosynthesis), biosynthesis of other secondary metabolites (Prodigiosin, Glucosinolate, Phenazine, and Flavonoids), Ketone bodies biosynthesis, Terpenoids metabolism (Terpenoid and zeatin biosynthesis), Glycan biosynthesis (O-Antigen repeat unit) and xenobiotic metabolism (Dioxin). Moreover, some cellular processes involved in translation (Aminoacyl-tRNA biosynthesis) and cell growth and death (Cell cycle – Caulobacter) also increased.

Consumption of the sterile cheese matrix did not present any significant difference when compared to the DSS Group (Fig. 5C and Table S5). Mice consuming the Single-strain cheese by contrast exhibited an enrichment of energy metabolism (carbon fixation pathways), carbohydrate metabolism (pyruvate metabolism), and transcription process (RNA polymerase) (Fig. 5D). However, purine nucleotide and taurine metabolism were reduced. When comparing DSS + Emmental and DSS groups, Emmental consumption increased the number of genes involved in cellular processes such as membrane transport (ABC transporter and phosphotransferase system), quorum sensing, and beta-lactam resistance.

Contrarily, Emmental consumption resulted in a reduction of peptidoglycan metabolism, flagellar assembly, and bacterial chemotaxis (Fig. 4 and Fig. 5E). Although non-significant, an increase in galactose, starch, and sucrose metabolism was also observed (Fig. 5E and Table S6).

Individual taxa contribution analysis and metabolic pathway reconstruction in mice treated with the Emmental-type cheese in healthy and in colitis conditions:

Akkermansia muciniphila **is a major contributor to nervous system functions in healthy mice**

Metagenome Assembled Genomes (MAGs) were recovered from the Emmental Group (sample B238) and taxonomically classified based on Tetra correlation analysis (TCA) against Jspecies database: *Akkermansia muciniphila* (97,9% Completeness, 0,0% Contamination); *Alistipes dispar* (77,2% Completeness, 6,4% Contamination) and *Muribaculum intestinale* (46,5% Completeness, 3,4% Contamination) (Table S7). The phylogenomic analysis has confirmed *Akkermansia muciniphila* taxonomy at the species level (Figure S4). MAGs contribution to biological pathways is shown in Fig. 6B and Figure S5. It was observed that the *M. intestinale* genome contains more genes, when compared with the other MAGs, which contribute to functions related to global, co-factor and vitamins, and energy metabolism (carbon fixation, Methane, and Nitrogen), except for sulfur metabolism genes, which were more present in *A. muciniphila* genome. Regarding amino acids metabolism, *A. muciniphila* has more genes contributing to Tryptophan metabolism and genes that interact with GABAergic synapse pathways. Moreover, genetic information processing related to translation and transcription was more enriched in this bacterium. *At. dispar* genome presented the lowest number of genes contributing to most of the functions.

The Integrated metabolic pathways map reconstruction suggested that the *A. muciniphila* genome presented most enzyme genes (13 from 16 genes) required for tryptophan biosynthesis from intermediate metabolites from glycolysis and pentose pathways. Moreover, *M. intestinale* and *At. dispar* can also contribute to 9 and two steps, respectively, in tryptophan biosynthesis (Fig. 6B). However, only *At. dispar* genome contained the tryptophanase enzyme gene [EC:4.1.99.1] required to produce Indole (Figure S6).

Regarding the GABAergic synapses pathway, the glutaminase [EC:3.5.1.2] and glutamate decarboxylase [EC:4.1.1.15] genes involved in GABA production by converting glutamate were exclusively found in the *A. muciniphila* genome. At the same time, the other bacteria only contributed to glutamine and glutamate biosynthesis from the citric acid cycle intermediate metabolites (Figure S6). Sulfur reduction assimilatory metabolism genes were exclusively found in the *A. muciniphila* genome, and the reconstructed map (Figure S6) suggests this bacterium can produce acetate from hydrogen sulfide and serine metabolism through the activity of cysteine synthase [EC:2.5.1.47] and serine O-acetyltransferase [EC:2.3.1.30].

Ligilactobacillus murinus **is a major contributor to central carbohydrate metabolism in mice microbiome population and presents genes involved in the production of metabolites with potential anti-inflammatory activity**

The TCA-based taxonomy of MAGs recovered from the DSS + Emmental Group (sample B266) was *Ligilactobacillus murinus* (54,0% Completeness and 2,4% Contamination); *Duncaniella dubossi* (46,5%

Completeness and 10,3% Contamination) and *Helicobacter apodemus* (46,0% Completeness and 9,1% Contamination) (Table S7). The *Lg. murinus* taxonomy was also suggested by the phylogenetic tree (Fig S4). MAGs individual contribution to biological pathways is shown in Fig. 7B and Figure S5. Regarding metabolism, global metabolic function genes were more abundant in *Lg. murinus*, followed by *D. dubossi* and *H. apodemus. Lg. murinus* was the major contributor to carbohydrate (starch and sucrose, galactose, fructose and mannose), Nucleotide (purine) metabolism. *D. dubossi* and *Lg. murinus* had an equal contribution to amino acids metabolism, although the first bacterium species genes were more related to phenylalanine, tyrosine, and tryptophan biosynthesis, while the late species genes were associated with alanine, aspartate, and glutamate metabolism. Energy metabolism genes were predominantly found in *H. apodemus* and primarily associated with oxidative phosphorylation. Most co-factors and vitamin metabolism genes were distributed between the genomes from *Lg. murinus* (vitamin B6 and thiamine metabolism, phanthenate and CoA biosynthesis), and *D. dubossi* (biotin, riboflavin, and folate metabolism). Regarding environmental and cellular processes, *Lg. murinus* genome contains more genes involved in membrane transport (ABC transporters), signal transduction (two-component system), and biofilm formation.

Reconstructed pathways maps have shown ABC transporters system in the *Lg.murinus* genome was most related to the transport of phosphate, amino acids (glutamine, cystine, Arginine, histidine, and lysine), oligopeptides, and inorganic ions (Figure S7). The phosphotransferase system in this bacterium is involved in the uptake of several sugars (glucose, mannose, and fructose), while *H. apodemus* presents only a fructose up-take system (Figure S7). Regarding metabolites with potential anti-inflammatory properties, all three bacteria contribute to acetate production via glycolysis/pyruvate metabolism, although most genes involved in central carbohydrate metabolism were detected in *Lg. murinus* genome (Fig. 7B). Moreover, the same bacterium contains glutamate-cysteine ligase [EC:6.3.2.2] required to produce glutathione (Figure S7). Although *D. dubossi* and *Lg. murinus* genomes presented most Co-factors and vitamin metabolism genes. Thiamine, riboflavin, and vitamin B6 pathways lack enzyme genes in their biosynthesis. Regarding lipid metabolism, *H. apodemus*, and *Lg. murinus* contribute to the biosynthesis of phosphatidylethanolamine through the metabolism of glycerophospholipids by phosphatidate cytidylyltransferase [EC:2.7.7.41] and CDPdiglyceride-serine O-phosphatidyl transferase [EC:2.7.8.8].

Ligilactobacillus murinus **presents potential immunomodulatory interaction with the human gut Immune system**

Lg. murinus presented 20 Cell wall proteins classified as adhesins. From those, 17 proteins were predicted to interact at least with one human protein involved in the inflammasome activation pathway, as shown in Fig. 8 and Table S8. Bacterial adhesins presenting the best interaction prediction score (100% identity with known interaction network proteins) were B266-M15-00085, B266-M15-00251, and B266-M15-01787. All three in silico interactions occurred with the NF-kB p105 human protein. In quantitative terms, most interactions occurred between bacterial proteins and NF-kB p105 (17 interactions), followed by NLRP1 and NAIP5 (11 interactions). InterPRO domain analysis suggests the presence of Mucin-type Glycan binding domains (data not shown).

Discussion

Homeostasis context

Consumption of both experimental kinds of cheese, in a healthy or colitis context, promoted distinct changes in mice microbial communities. The present study revealed that five-day diet complementation based on the control sterile cheese matrix, on a Swiss-type cheese exclusively fermented by *P. freudenreichii* CIRM-BIA129 or on an Emmental cheese manufactured using *P. freudenreichii* CIRM-BIA129 in association with *L. delbrueckii* CNRZ327 and *S. thermophilus* LMD-9 as starters, does not cause long-term shifts of the gut microbiota. In particular, no unbalanced composition (dysbiotic) profile was observed in healthy mice. In this context, we observed that microbial diversity was kept, and most of the taxa found in the normal microbiome exhibited no significant changes in population sizes after the three complementations. Moreover, PCA and ANOSIM analysis regarding KEEG functional profiling support these findings and suggested that none of the experimental cheeses impaired fundamental microbiota functions. Therefore, they might be safe for healthy consumers.

Considering the effects promoted by the sterile cheese matrix composed of an acidified curd-like mixture of cow milk fat, protein, and carbohydrate, the microbiota cellular process involved in genetic information processing like Sulfur relay system, RNA polymerase, and phosphatidylinositol 3' -kinase(PI3K)-Akt signalling functions were found enriched. One factor associated with the modulation of such functions is the cheese matrix's acidic (pH5.5) condition. In this context, gut microorganisms with greater capacity to adapt and survive in this environment could have expanded, such as the increase in Sulfur relay system genes involved in many Post-transcriptional RNA modifications [11]. Moreover, the gene mapping in the PI3K-Akt signalling pathway was predicted as a heat shock protein chaperone when aligned to Uniprot Protein DB (98,02% Identity, A0A1B1SDF3), hence, supporting the stress adaptation hypothesis. It was also expected that the cheese matrix substrate could work as a prebiotic factor for the gut bacteria, as suggested previously [12]. These genes were also increased in mice receiving the Emmental type cheese. In this case, the acidification of the cheese promoted by the production of propionic and acetic acid, as a result of propionibacteria fermentation could be involved in selecting low pH stress tolerating mechanisms.

Swiss-type cheeses are usually high-fat, and therefore the excess of lipids could provide a niche for other lipolytic bacteria in the gut [10]. Consuming cheese containing only *P. freudenreichii* CIRM-BIA129 induced the enrichment of lipid metabolism capacities, such as the increase in Fatty acid biosynthesis genes and a decrease in the biosynthesis of LPS. This effect was not observed in mice consuming the Emmental-type cheese containing the thermophilic Latic Acid Bacteria strains, suggesting that their lipolytic activity might complement propionibacteria in the breakdown of lipids. The impact of the enrichment in lipolysis promoted by commensal bacteria in the host functions is largely unknown and should be further investigated. On the other hand, LPS are glycans produced by Gram-negative bacteria and have been strongly associated with the stimulation of pro-inflammatory responses in the gut [9]. Our study found the LPS biosynthesis pathway incomplete in the normal microbiota. The number of genes in

this pathway was further reduced after treatment with the cheese containing only *P. freudenreichii* CIRM-BIA129, suggesting this approach could help prevent the initiation of an inflammatory process.

Xenobiotics degradation genes were predicted within the gut microbiome of control mice and of mice consuming the Emmental cheese. An increase in Benzoate metabolism genes was observed after cheese consumption. However, other xenobiotics metabolism genes, such as styrene and caprolactam, were absent in these animals. Sodium benzoate is widely used for the preservation of fermented foods, including cheeses. It is furthermore produced from hippuric acid and phenylalanine metabolism because of bacterial fermentation in cheese [13]. Studies have pointed out that frequent exposure to xenobiotics from the diet may have allowed gut microbes to evolve the capacity to metabolize them [14], [15].

In contrast to the Single-strain cheese, the Emmental-type cheese was prepared following an industrial scale process, which could explain the increase in xenobiotic degradation. Natural preservatives in the food industry are replacing Sodium Benzoate, and we suggest this improvement should be considered for the development of safer functional foods as these metabolites have been associated with toxic effects when consumed in high concentrations [16]. Another crucial metabolic function affected by the experimental Emmental cheese consumption in healthy mice was the decrease in Amino sugar and nucleotide sugar metabolism. This pathway involves many glycation processes of lipids and proteins in bacteria, including peptidoglycan or O-antigen (LPS) biosynthesis. Pathway reconstruction analysis suggests the microbiome of Emmental treated mice kept the ability to phosphorylate D-galactose and D-glucose but lacked the D-hexose 6-phosphotransferase gene, indicating a limited capacity regarding sugars like mannose and fructose (data not shown). This effect could result from a low-carbohydrate diet as most of the milk sugar is probably consumed by the starter bacteria used for cheese fermentation.

Sulfur metabolism is critical in the host and microbiota functions, including physiologic and pathogenic ones. Sulfate substrates can be found in the gastrointestinal tract in the inorganic form or conjugated with organic substances such as mucin proteins, bile acid, and amino acids from the diet [14]. The present report suggests a decrease in sulfur metabolism, caused by Emmental consumption, as indicated by a lack of genes involved in the uptake of inorganic Sulfate. The gut microbiota however kept the ability to obtain sulfate from Sulfonated organic compounds. Interestingly, this result corroborates with the partial increase in *A. muciniphila*, which has been reported as a probiotic mucin scavenger bacterium in the gut [17]. Furthermore, two kinds of sulfate-degrading microorganisms have been described in the bacteria kingdom: (i) Sulfidogenic, such as *Desulfovibrio* spp. or *Bilophila* spp, which use sulfate as a final electron acceptor to generate energy and produce inorganic hydrogen sulfide (HS) as an end-product that can be toxic when produced in large amounts and has been associated with IBD pathogenesis [18]; (ii) Sulfate assimilatory, including *A. muciniphila*, that consume energy to incorporate Sulfate in several organic compounds, including peptides and amino acids and may produce secondary metabolites which can be used as a carbon source for other commensal bacteria [19]. The *A. muciniphila* genome recovered from a metagenome sample (B238) from a mouse consuming Emmental revealed a complete Sulfur assimilatory pathway. Moreover, pathway contribution analysis supported beneficial properties modulated by Emmental consumption, as it was shown this *Akkermansia* along with Bacteriodales

members, *Alistipes*, and *Muribaculum*, may produce acetate via sulfur metabolism, Indole and GABA via amino acids degradation, all considered key metabolites in the regulation of the host immune system in intestinal and gut-brain axis inflammatory disorders [20], [21].

Previous studies have reported that proteolysis of Casein, milk's most abundant protein, can produce bioactive Glycomacropeptides (GMP) containing mucin-type carbohydrate chains such as N-acetylgalactosamine and N-acetylneuraminic acid [12]. Milk mucin-type GMPs are rich in galactose, glutamate, and serine. They, therefore, could provide the primary substrate for *A. muciniphila* to produce metabolites with neuromodulatory properties such as Indole, GABA, and Acetate, respectively, as suggested by pathway reconstruction and contribution analysis performed in our study. A prebiotic formulation that increased *Akkermansia* based on sialyl glycopeptide (SGP) extracted from milk GMP was recently developed and patented (JP2019043867A) in Japan. These results together suggest that the synergic proteolytic activity of the thermophilic bacteria *L. delbruekii* and *S. thermophilus* in the fermentation process of Emmental cheese might have a crucial role in the enrichment of *A. muciniphila* in the gut. Therefore, we suggest that selecting probiotic strains with appropriate proteolytic activity, generating GMP might be the key to developing functional dairy foods to promote the enrichment of this bacterium in the gut.

Another common member of the typical mammalian microbiota, *Romboutsia*, was increased around ten times when mice consumed the Emmental-type cheese. Interestingly, this genus has been found enriched in rat microbiota after receiving a mix of lactobacilli probiotic strains [22]. This genus is an anaerobic member of the Firmicutes phylum and is closely related to *Clostridium sp.* Its role in the gut microbiota is poorly understood. However, it is known that the primary metabolic end-products of the type species *R. idealis* are SCFA, acetate, and formate when cultivated in artificial media [23]. We could not investigate the presence of the possible genes associated with SCFA biosynthesis in this bacterium due to the small number of reads obtained.

Inflammation Context

DSS administration did not affect the microbiota diversity regardless of the type of cheese treatment. It did, however, modify the gut population of genera *Bacteroides* and *Parabacteroides*, as well as the gene content as expected of imbalanced community shifts found in IBD patients [22]. Carbohydrate metabolism was the most affected KEEG pathway, with fewer genes in the context of colitis. Among the pathways that conversely exhibited an enrichment, the O-Glycan biosynthesis pathway was noticed. The concomitant increase of Gram-negative bacteria such as *Helicobacter* ssp can explain this finding.

Interestingly, PCA and ANOSIM revealed functional dissimilarity between the DSS control group and groups of inflamed mice consuming cheese, suggesting different signatures in microbiome structures. While cheese matrix consumption has not shown any improvement in structural and functional aspects, our results suggest that consuming single-strain cheese could restore some of the impaired metabolic functions related to carbohydrate metabolism, including pyruvate and carbon fixation pathways.

However, it could not contain the growth of *Helicobacter* spp., which is considered an opportunistic commensal in rodent gut [24]. This finding is intriguing as we have previously reported that the cheese fermented exclusively with *P. freudenreichii* could ameliorate inflammatory markers in murine DSS-induced colitis, such as reduced TNF and IFN-gamma [4]. LPS (O-antigens) from *Helicobacter* spp. possess weak pro-inflammatory effects but are vital for the persistence of the bacterium by providing camouflage as it mimics glycans present in the gastrointestinal mucosa [25]. We hypothesize that despite the anti-inflammatory properties of *P. freudenreichii*, the prebiotic factors and environment acidification of the cheese could have allowed the expansion of *Helicobacter* as this microorganism presents resistance mechanisms such as Urease coding genes for tolerating low pH [24].

In contrast, the consumption of the Emmental-type cheese enhanced the intestinal population of the lactobacilaceae *Lg. murinus.* This bacterium has shown promising probiotic properties in treating intestinal inflammation disorders; and a recent study has reported its ability to alleviate DSS-induced colitis in mice [19], [26]. Another crucial functional impact of Emmental-type cheese consumption in mice with DSS-induced colitis was the increased diversity of sugars and amino acids uptake systems (ABC transporters systems and phosphotransferases systems) found in *Lg murinus* MAG, suggesting an improved capacity to adapt and assimilate different kinds of nutrients. While *Duncaniella* spp. MAG shares many genes in common involved in global metabolism, *Lg. murinus* MAG presented a higher abundance of genes involved in producing metabolites with potential anti-inflammatory activity, such as acetate and glutathione.

Furthermore, PPI analysis suggested the ability of *Lg. murinus* cell wall layer adhesin proteins to exert an immunomodulatory activity by interacting with human and mouse Inflammasome sensor proteins, mainly NLRP1 and NAIP5. Additionally, the NF-kB p105 subunit is predicted to interact with several ligands from this bacterium cell wall. Commensal probiotic strains have been reported to possess immunomodulatory properties related to NLRP1 and NAIP5 inflammasome attenuation and regulation of inflammation markers by inhibiting the NF-kB complex formation [27]–[29]. These findings corroborate our previous study, showing that the Emmental cheese diet reduced the expression of IFN-gamma and TNF and prevented tissue damage caused by DSS in mice [4]. This might explain the mechanism behind its beneficial properties. However, further investigations are required to confirm whether these interactions involve inhibitory or activation mechanisms. In addition, these results suggest that *Lg. murinus* adhesins may provide competition with other luminal ligands as toxins and antigens from commensal pathobionts such as *H. apodemus.*

Conclusions

This study concludes that experimental Emmental-type cheese is safe for healthy consumers and may promote the increased capacity of the microbiota to produce metabolites with neuromodulation properties in gut-brain axis regulation. However, further studies are required to fully characterize the impact of cheeses on the central nervous system and neurodegenerative diseases. Furthermore, our

results suggest possible anti-inflammatory mechanisms based on the interaction of enriched commensal *Lg. murinus* with the gut immune system.

Material And Methods

Cheeses preparation and bacterial strains

Cheeses were manufactured as described in a previous study [4]. For a control sterile matrix preparation, UHT cow milk was added with milk proteins and cream before acidification using Glucono Delta Lactone, moulding, pressing, drying, and wrapping. An experimental single-strain cheese was prepared the same way following the growth of *Propionibacterium freudenreichii* CIRM-BIA129 in UHT milk. Finally, an Emmental cheese was manufactured at an industrial scale by the Entremont alliance company (Malestroit, France). *Lactobacillus delbrueckii* subsp *lactis* CNRZ327, *Streptococcus thermophilus* LMD-9 and *P. freudenreichii* CIRM-BIA129 were used as starters in this industrial Emmental cheese. All bacterial strains were provided by the international microbiological resource centre CIRM-BIA (Centre International de Ressources Microbiennes, Bactéries d'Intérêt, France). A sterile mixture of cow milk, milk cream, and casein peptone was used as the substrate matrix for starter bacteria. For the placebo control, a solution of Glucono Delta Lactone was used to reproduce acidification during fermentation (pH 5.5).

Animal Experiments And Sample Collection

The present study used the same mice as previously in Rabah and Colleagues (2018). Female C57BL6 mice between six and eight weeks were obtained from the Federal University of Minas Gerais (Belo Horizonte, Brazil). The study was approved by the Brazilian Ethics Committee on Animal Use (CEUA, Brazil, protocol 364/2018), and the animals were housed in a 12 h/12 h light/dark cycle-controlled room with a temperature of 25°C and unrestricted access to standard chow and filtered water before the experiment. We, at this moment, confirm that the study was carried out in compliance with the ARRIVE guidelines.

For the *in vivo* assay, a colitis preventive treatment protocol was followed, as shown in Fig. 9. Mice were divided into eight groups (n = 6). The first four groups consisted of noninflamed mice pre-treated with probiotic cheeses for five days: (i) the negative control (Naïve group), which did not receive any treatment; (ii) the Matrix group, receiving 400 mg of a sterile nonfermented cheese matrix daily by gavage; (iii) Single-strain and (iv) Emmental groups which received daily gavage with 400 mg of cheeses prepared as described above.

The remaining four groups were composed of mice pre-treated with probiotic cheeses for five days and challenged with colitis induced by drinking 3% dextran sulfate sodium (DSS) (MP Biomedicals, Illkirch-Graffenstadenn, France) during seven days: (v) DSS control group, mice receiving DSS treated with PBS (Phosphate-Buffered Saline); (vi) DSS-Matrix group, receiving DSS and fed with the nonfermented cheese matrix; (vii) DSS + Single-strain and (viii) DSS + Emmental groups, mice receiving DSS and fed by gavage

either with experimental or conventional Emmental cheeses. All mice were euthanized on the 12th day of the experiment, followed by stool samples collected and stored at − 80°C.

Metagenomic Dna Isolation And Sequencing

Total DNA was extracted from 48 mice stool samples (100 mg) following QIAamp DNA Stool Mini Kit protocol (Qiagen) recommendations and used to prepare whole shotgun metagenomic libraries performed with the Illumina TruSeq DNA Sample Preparation kit. The Metagenomic DNA libraries were sequenced using the HiSeq 2500 platform (Illumina) with paired-end (2 × 150 bp) reads with inserts size of 450bp. Sixteen samples could not be sequenced due to the low yield of high-quality DNA. A summary of the amounts of reads obtained for each sample is shown in Table S9.

Public Genomes Used In This Study

Datasets of 97 complete public genomes of *Akkermansia spp.* strains and 17 *Ligilactobacillus* representative species were downloaded from the NCBI RefSeq genome database (accessed Sept. 23, 2022) for the phylogenomic analyses. The accession number of all genomes is shown in Table S10.

Microbiome Taxonomy And Function Profiling

FastQ file base quality was verified using FastQC v.0.11.9 tool, and the low-quality reads (Phred < 30) were filtered using Fastp v. 0.232 software. A 150pb read-based taxonomic classification was performed using kaiju v1.9.2 software [30], aligning the sequences to the NCBI BLAST nr + euk sequences database. Afterwards, the relative abundances of bacterial taxa in the samples were estimated and statistically compared using RStudio built-in functions and STAMP v2.1.3 software. A diversity analysis was conducted by estimating the Shannon index using the Vegan package for RStudio.

The reads classified as Bacteria were extracted and submitted for a de novo assembly using the megahit v.1.2.9 tool for functional analysis. Following the assembly, contigs were annotated using Prokka v1.14.5. An enrichment analysis based on orthologs was performed using the KEGG database, and the number of genes for each category/pathway was compared statistically among the groups using the same tools described above.

A normality test was performed using the Shapiro-Wilk test for the comparative analyses performed among the groups (n = 6). ANOVA or Kruskal-Wallis's test was used for data presenting either Gaussian or asymmetric distribution, respectively, followed by Tukey or Welch's T-test for Post hoc analysis. A Chi-square hypothesis test was used to compare data from the DSS-Emmental group (n = 1) to other groups. For Principal Component Analysis (PCA) data, an Analysis of similarities (ANOSIM) was performed. Significant differences were considered when the P-value < 0,05.

Mags Reconstruction And Individual Taxa Contribution Analysis

To evaluate the individual functional contribution of taxa, *de novo* assembled contigs (minimum length = 1000pb) from one sample selected from the group Emmental and one sample from the DSS-Emmental group was used for recovering metagenome-assembled genomes (MAG) based on a binning algorithm performed by the MaxBin2 v. 1.2.0 tool [31]. The MAG completeness and contamination were verified using CheckM v1.2.2 software. The taxonomy at the species level was investigated by tetranucleotide pairwise alignment against the JSpeciesWS tool genomesDB database followed by a multi-locus (92 single copy orthologous genes) phylogeny using the Up-to-date bacterial core gene (UBCG) v. 3.0 tool. The trees were built using the Maximum Likelihood method with 1000 Bootstrap replicates. For this step, it was used a dataset of complete public genomes described in item 2.4. Afterwards, the number of genes per KEGG pathway for each MAG was estimated, as described above, compared, and clustered in a heatmap plot using the Pheatmap package for Rstudio. Then, the list of genes from all MAGs was mapped to KEGG pathways using the KEEGmapper tool. All the maps (map00620, map04727, map00400, map00380, map00250, map00920, map02010, map02060, and map00480) used for the reconstruction and visualization of MAGs integrated metabolic pathways were initially generated by Kanehisa laboratories [32].

Identification Of Mags Cell-adhesion Related Sequences And Protein-protein Interaction With Human Gut Inflammatory Pathways

Translated amino acid sequences from the MAG B266-M15 (*Ligilactobacillus murinus*) were submitted to the PsortB (https://doi.org/10.1093/bioinformatics/btq249) v3.0 (accessed on 02/02/23) web-platform for extraction of proteins located on Cell Wall surface. Subsequently, all cytoplasmic proteins were submitted to Vaxign2 (https://doi.org/10.1093/nar/gkab279) (accessed on 02/02/23) to select adhesin-related proteins, considering a 60% probability cutoff. Human, mice, and homologous pig proteins were discarded. Afterwards, Protein-Protein Interaction (PPI) was performed with human inflammasome key proteins (https://doi.org/10.1038/mi.2017.19, https://doi.org/10.3390/microorganisms9040829). The GenBank ID for the 17 selected proteins is available in Table 1. For this step, cell adhesion-related proteins were submitted on INTERSPPI - Human&Bacteria v3 (10.1021/acs.jproteome.9b00074) (accessed on 02/03/23) to predict PPI, considering a 97% of identity against the platform databases and 95% interaction probability cutoff. Networks were plotted in the Rstudio igraph package and Python networkx and pyvis packages.

Table 1
Inflammasome pathways-associated proteins in this study.

Protein	GenBank ID
NLRP1	NP_127497.1
NLRP3	NP_001230062.1
NLRP6	NP_612202.2
NLRP12	NP_653288.1
NLRC4	NP_001186067.1
AIM2	NP_004824.1
NAIP5	Q9R016.3
NAIPs	AAI36274.1
NFk-B_p105	NP_003989.2
TRAF6	NP_004611.1
IRAK1	NP_001560.2
IRAK4	NP_057207.2
TLR4	NP_612564.1
NKFPBIA	NP_065390.1
RELA	NP_068810.3
TGFB1	NP_001278963.1
IKKB	O14920.1

Declarations

Ethics approval and Consent to participate

The study was approved by the Brazilian Ethics Committee on Animal Use (CEUA, Brazil, protocol 364/2018. We, at this moment, confirm that the study was carried out in compliance with the AVMA guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The experimental data and the simulation results that support the findings of this study are available in Figshare with the identifier https://doi.org/10.6084/m9.figshare.22495765. SRA data were deposited into NCBI under Bioproject ID PRJNA951221.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Rodrigo Carvalho: Investigation, Formal analysis, Visualization, Methodology, Writing - original draft, review & editing. **Houem Rabbah**: Investigation, Methodology. **Filipe do Carmo**: Investigation, Methodology. **Juan Ariute:** Formal analysis, Visualization. **Flávia Aburjaile**: Supervision, Writing - review & editing. **Bertram Brenig**: Resources, Methodology. **Yves Le Loir**: Funding acquisition, Project administration. **Eric Guédon:** Writing - review & editing. **Gwénaël Jan:** Conceptualization, Supervision, Writing - review & editing. **Vasco Azevedo**: Project administration, Resources, Supervision, Funding acquisition, Writing - review & editing.

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Figures

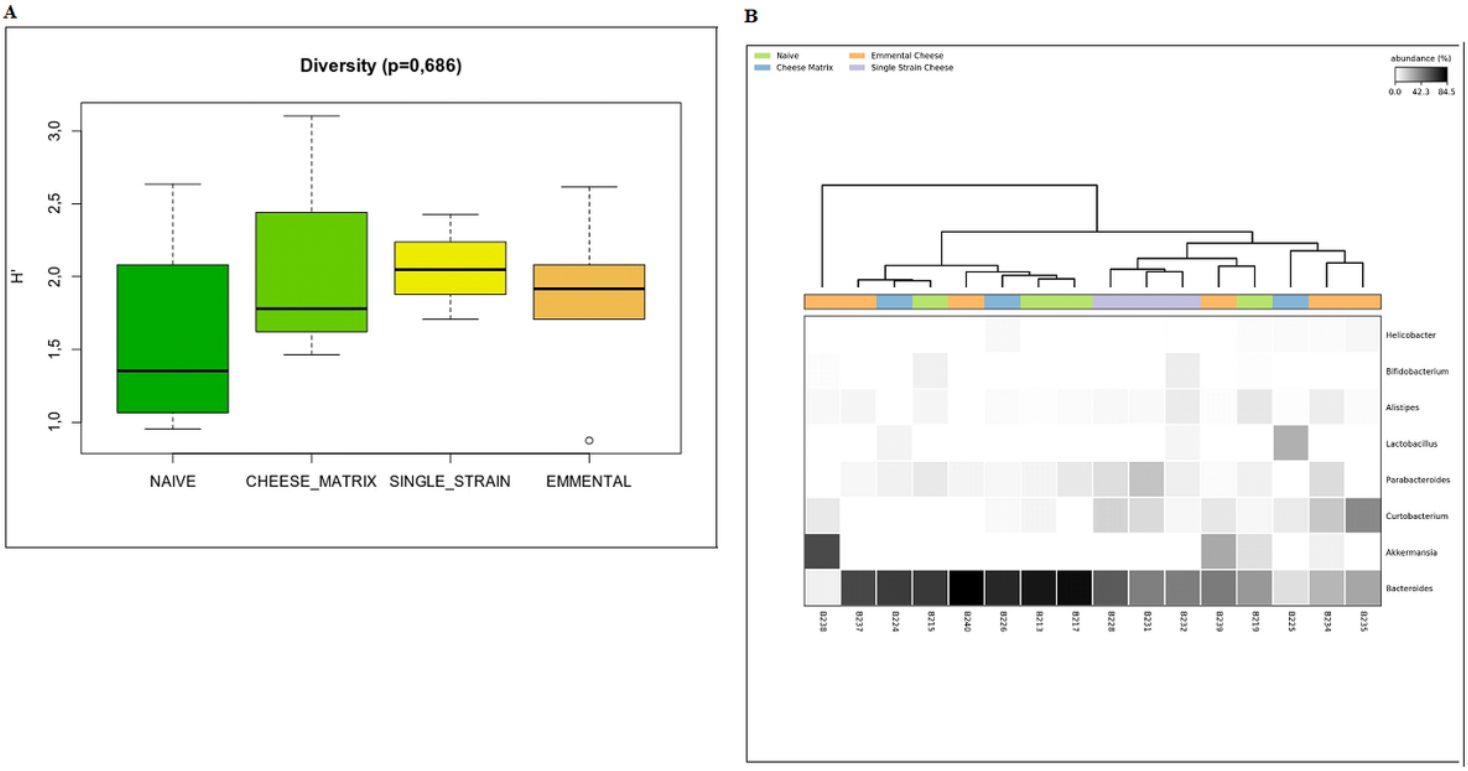

Figure 1

Microbiome taxonomy profile in healthy mice treated with cheeses. Beta-diversity analysis based on Shannon index (H’) in (**A**). The line inside the box plot represents the median of H'. The length of the whiskers represents 1,5 times the inter-quartile range (H'±IQR×1.5). The white circle in the graph represents outliers (>1,5xIQR). One-way ANOVA followed by Tukey Pos hoc test. No statistical significance, P-value > 0.05. Heatmap of bacterial taxonomy relative abundance in (**B**). Data in the graph represent the proportion of reads assigned to a given genus-level taxon. Dendrogram represents a hierarchical clustering based on the UPGMA method.

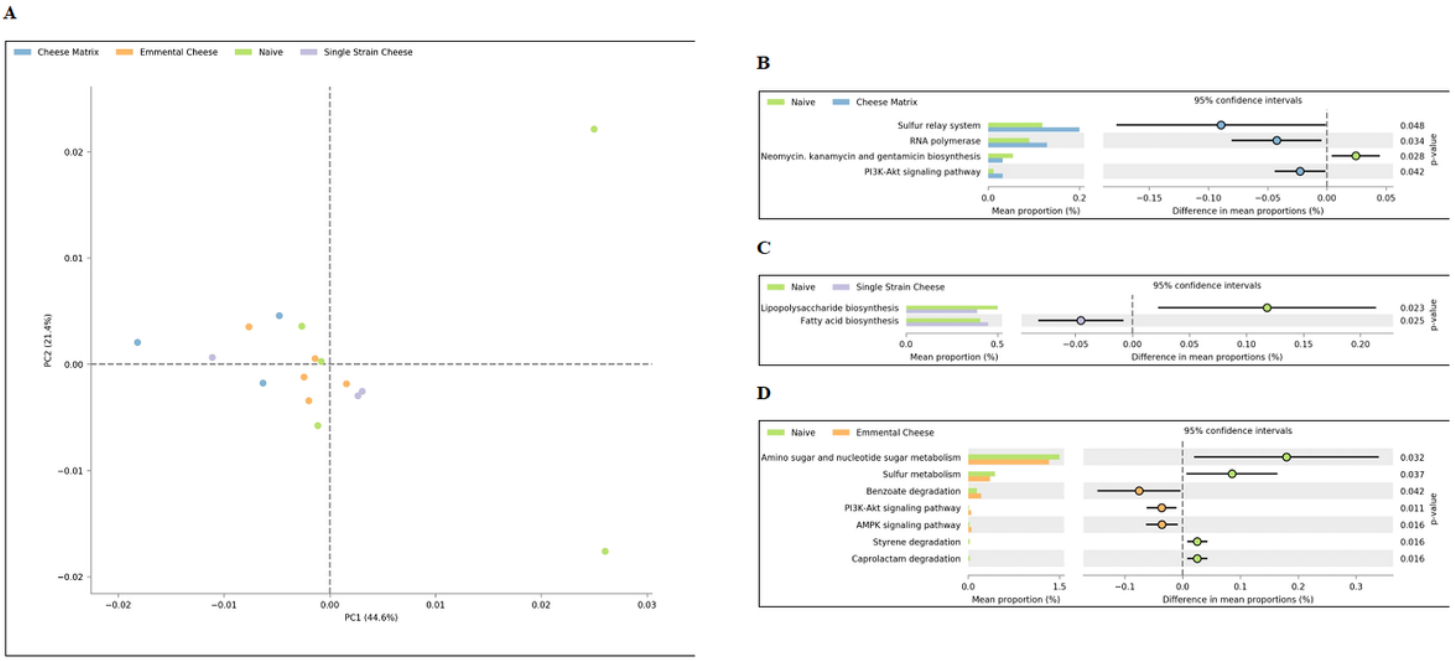

Figure 2

Microbiome functional profile in healthy mice treated with cheeses. Principal component analysis (PCA) plot of the number of genes per KEGG pathway in (**A**). Extended error bar plot showing the differentially enriched pathways between the following groups: Naïve and Cheese matrix in (**B**); Naïve and Single-strain cheese in (**C**), and Naïve and Emmental-type cheese in (**D**). Welch's test. Statistical significance when P-value < 0.05.

A

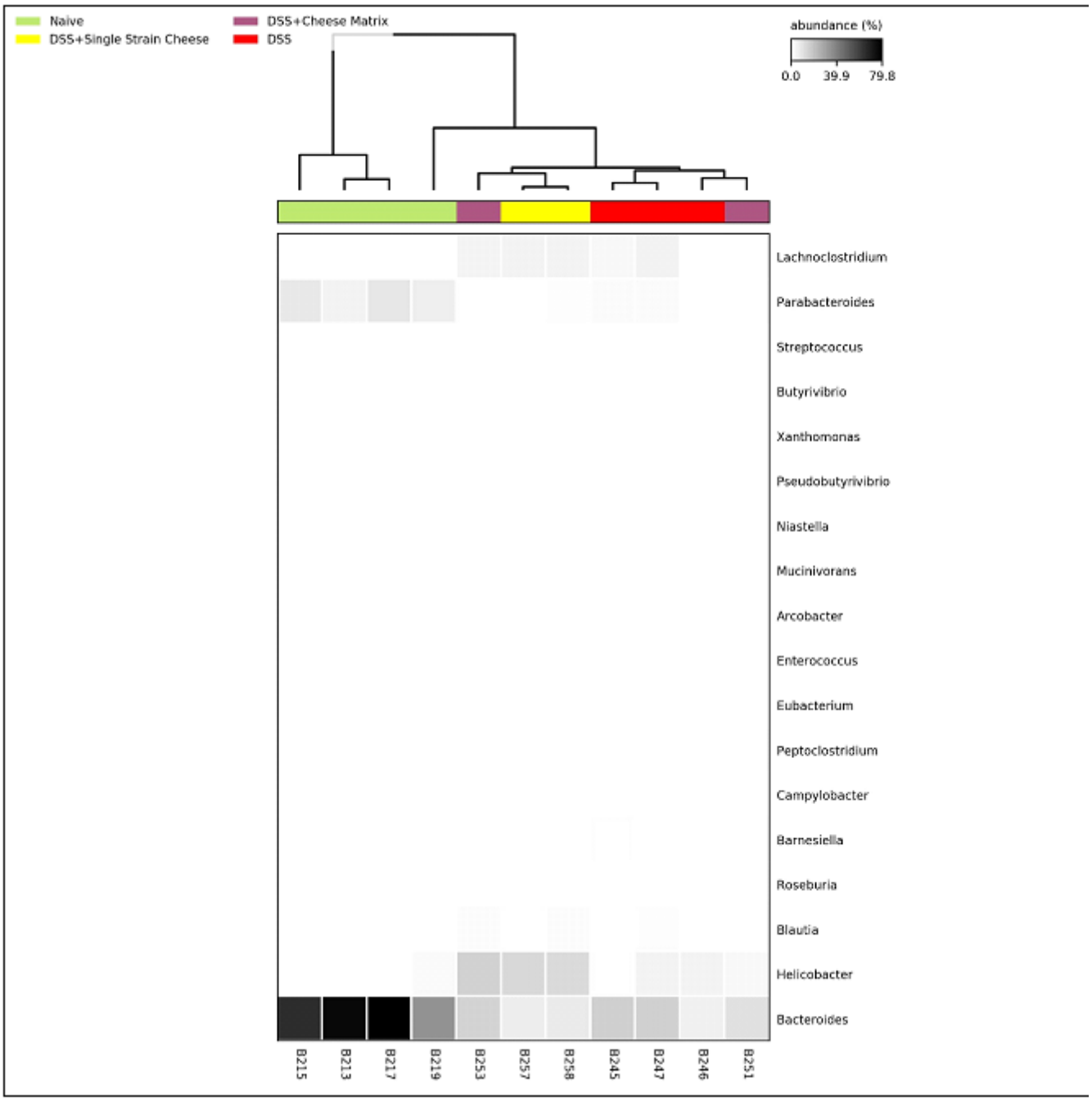

Figure 3

Heatmap of microbiome taxonomy profile in mice with colitis treated with the Single-strain cheese. Data in the graph represent the proportion of reads assigned to a given genus-level taxon. Dendrogram represents a hierarchical clustering based on the UPGMA method.

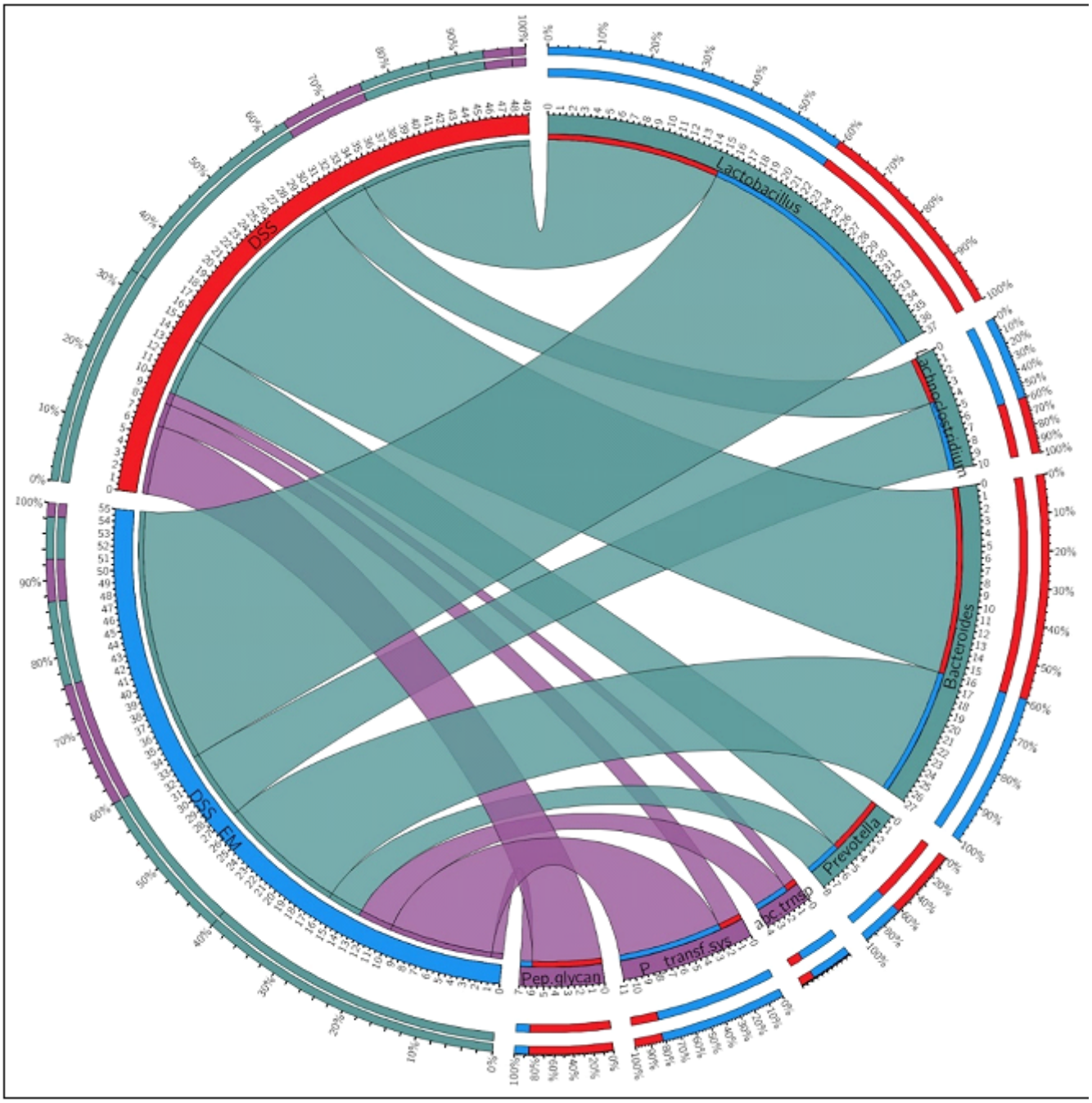

Figure 4

Circos plot representing the most enriched taxons and KEGG pathways in mice with colitis treated with Emmental-type cheese. Data in the graph represent the proportion of reads from samples B246 (DSS group) and B266 (Emmental-type cheese Group) assigned to a given bacterial genus in greyish blue. In purple is the proportion of genes mapped in each KEGG pathway. abc.tnsp stands for ABC Transporters, P_transf.sys for Phosphotransferase system, and Pep.glycan for Peptidoglycan metabolism. Chi-square test: All data represented in the graph were statistically significant ($p < 0.05$).

Figure 5

Microbiome functional profile in mice with colitis treated with cheeses. PCA plot of the number of genes per KEGG pathway in (**A**). Extended error bar plot showing the differentially enriched pathway between the following groups: Naïve and DSS in (**B**) DSS and DSS+Cheese matrix in (**C**); DSS and DSS+Single-strain cheese in (**D**); DSS (B246) and DSS+Emmental-type cheese (B266) in (**E**). Welch's test or Chi-square test. Statistical significance when P-value < 0.05.

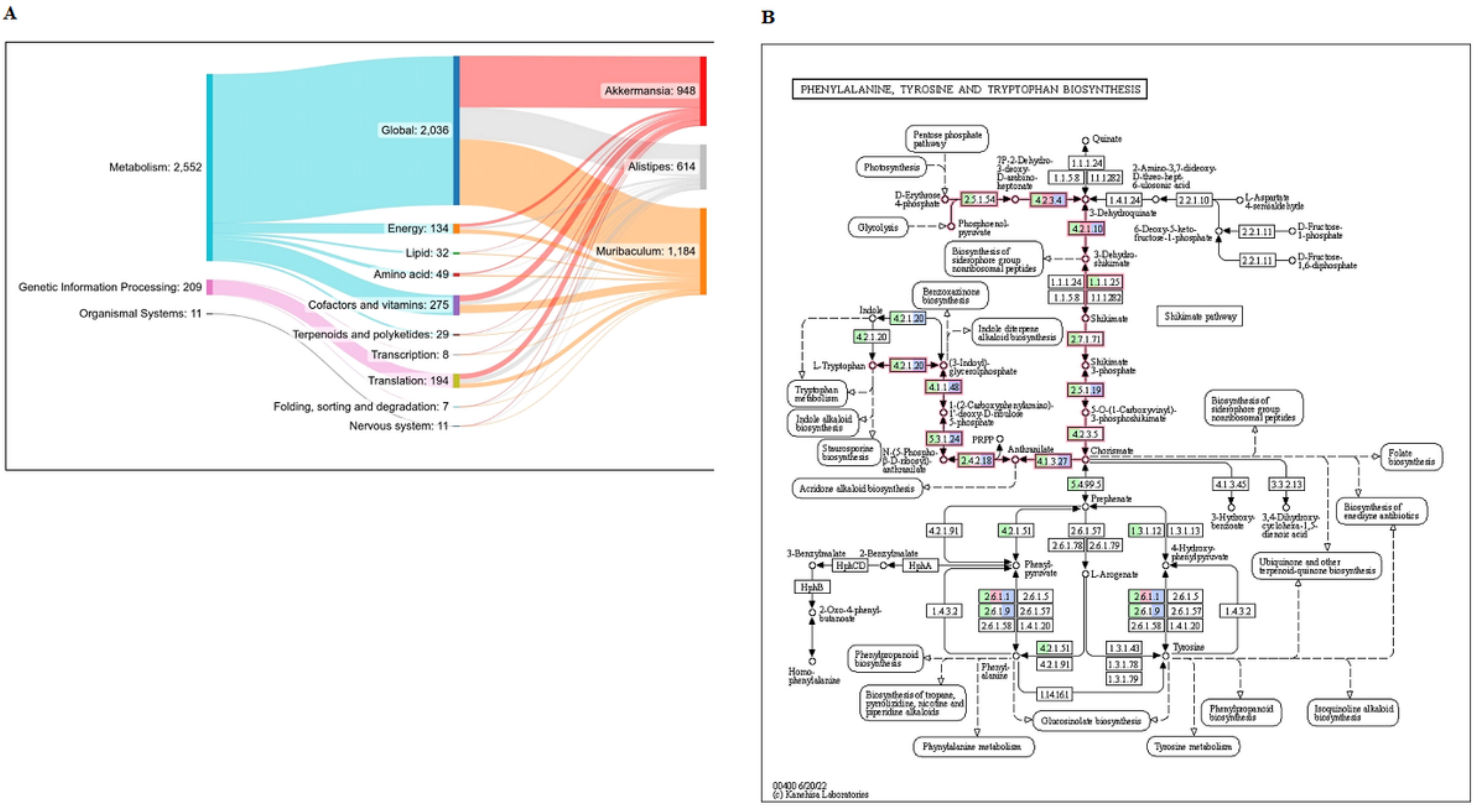

Figure 6

Functional contribution analysis and metabolic pathway reconstruction of MAGs recovered from the Emmental-type group.(**A**) Sankey diagram representing MAGs individual contribution in the number of genes to each KEEG pathway levels 2 and 3 categories. (**B**) MAGs Tryptophan metabolism integrated pathway. The EC number boxes' colors represent the taxa: *A. mucinlphila* in green; *At. dispar* in pink and *M. intestinale* in blue. Highlighted arrows indicated complete modules.

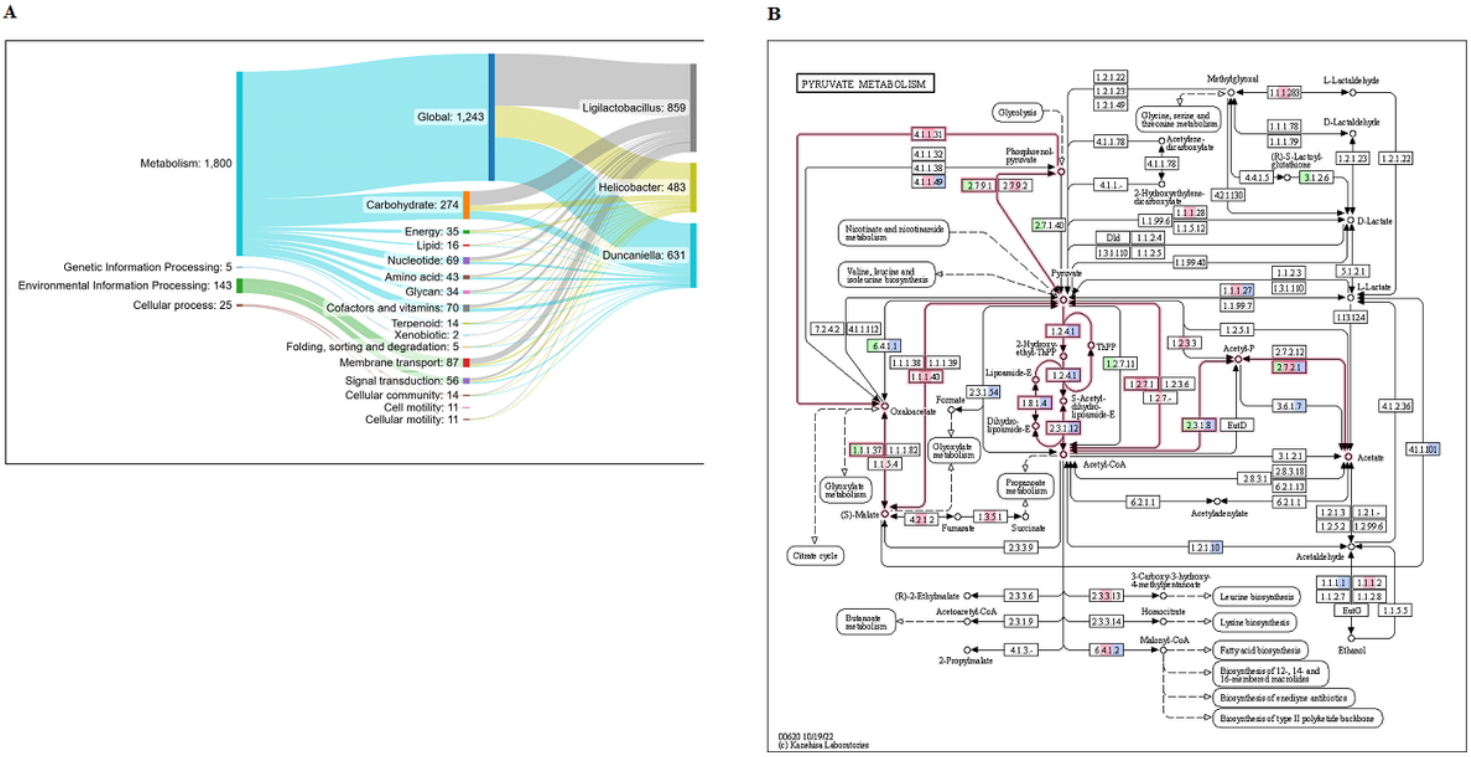

Figure 7

Functional contribution analysis and metabolic pathway reconstruction of MAGs recovered from DSS+Emmental group. (**A**) Sankey diagram representing MAGs individual contribution in the number of genes to each KEEG pathway levels 2 and 3 categories. (**B**) MAGs Pyruvate metabolism integrated pathway. The colors inside EC number boxes represent the following taxa: *D. dubossi* in green, *H. apodemus* in pink, and *Lg. murinus* in blue. Highlighted arrows indicated complete modules.

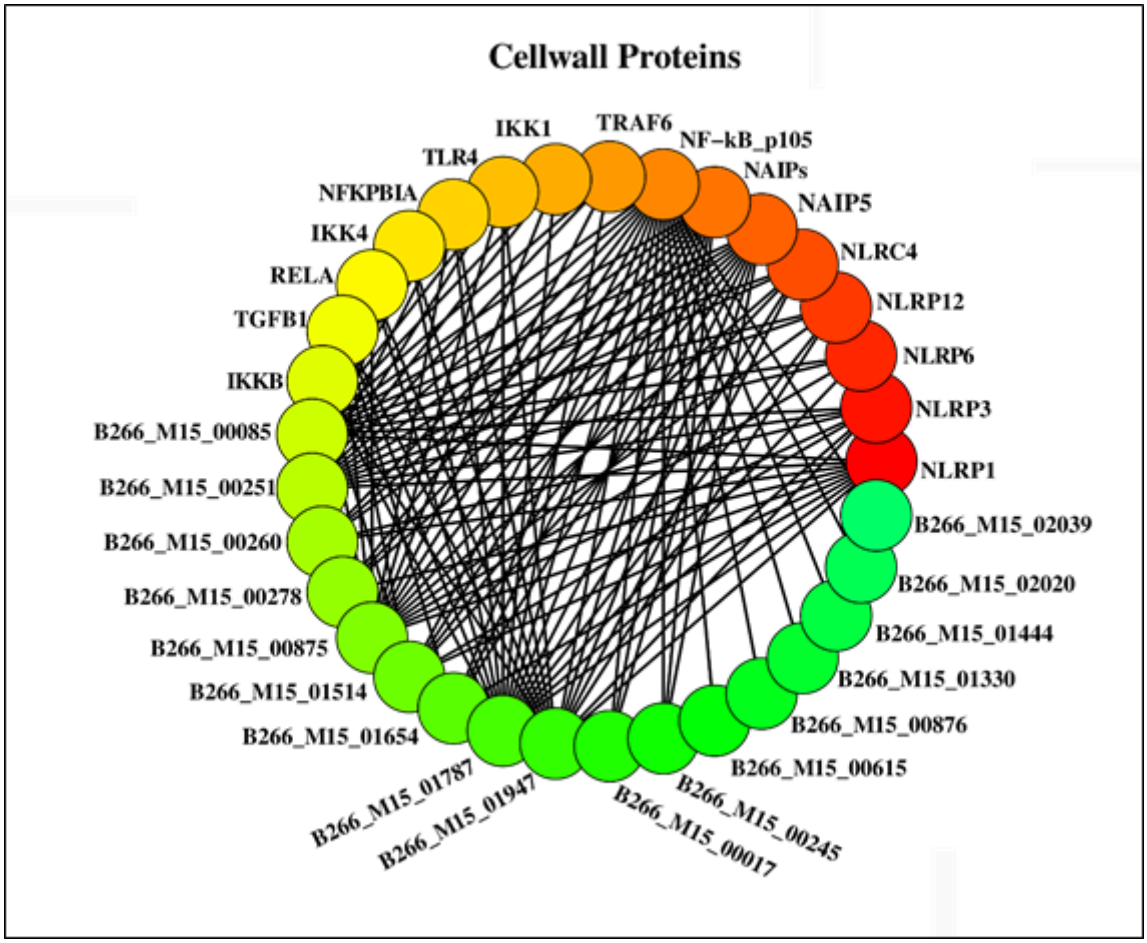

Figure 8

Protein-Protein Interaction network between *Lg. murinus* and human Inflammasome activation pathway. The nodes represent human or bacterial proteins, and lines connecting the nodes indicate interactions. B266-M15 captions represent *Ligilactobacillus murinus* MAG predicted proteins from sample B266 (DSS+Emmental Group).

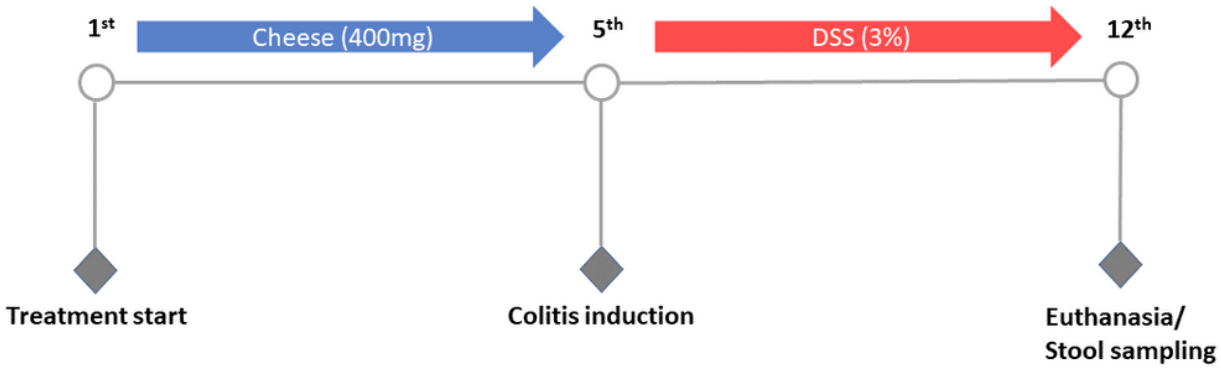

Figure 9

Colitis pre-treatment in C57BL6 mice with experimental cheeses. Intragastric gavage was administered to the cheeses daily (400mg of cheese in 500 µl PBS pH 7.4). DSS 3% solution was administered daily in drinking water. 100mg of mice stool samples were collected after euthanasia.

Supplementary Files

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