Characterisation of Tissue Damage in a Mouse Model for Pelvic Radiotherapy and Safety Assessment of two Potential Mitigators, *Limnospira indica* PCC8005 and *Lacticaseibacillus rhamnosus* GG ATCC 53103

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Running title: *Food Supplements in Pelvic Radiation Disease*

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ABSTRACT

Radiotherapy is a cornerstone treatment for pelvic cancer. However, gastrointestinal (GI) complications can occur as a result of collateral damage to healthy GI tissue. Recently, studies have linked gut microbial dysbiosis, i.e. an imbalance in microbial communities, to the development and progression of pelvic radiation disease. Consequently, scientists have been exploring adjuvant therapies for restoration of bacterial homeostasis or prevention of dysbiosis, including the use of microbial food supplements. The present study aimed to assess the GI damage induced by pelvic radiation (PR) and evaluate the safety of *Limnospira indica* PCC8005 and *Lacticaseibacillus rhamnosus* GG ATCC 53103, two potential mitigators of radiation-induced damage and dysbiosis. To investigate this, healthy C57BL/6J mice were locally irradiated and intestinal tissues were analysed. Additionally, healthy, non-irradiated mice were supplemented with *Limnospira* or *L. rhamnosus* and their organs were harvested to assess microbial supplement safety. Our findings suggest that PR induces inflammation in GI tissues, accompanied by infiltration of neutrophils and a reduction in colon length. In addition, PR causes atrophy in extraintestinal organs such as the spleen and bacterial translocation to mesenteric lymph nodes. Lastly, supplementation of food supplements to healthy mice revealed no adverse effects on different organs in the abdominal cavity. An increase in spleen size was seen in *Limnospira* supplemented mice, indicating its potential immunomodulating activities. Further research is warranted to confirm overall safety of these food supplements and explore their potential as radioprotective agents to improve health outcomes in pelvic cancer patients that undergo radiotherapy.

INTRODUCTION

Cancer is one of the leading causes of death worldwide (1). Pelvic cancers (e.g. colorectal cancer, ovarian cancer, prostate cancer) are among the most frequently diagnosed cancers (2). Radiotherapy plays a pivotal role in both curative and palliative management of pelvic cancers (3). Patients are usually treated according to a fractionation regimen of up to 60 Gray (Gy), in daily fractions of 1.8-2 Gy over a period of 4-8 weeks (4-6).

During radiotherapy, ionising radiation interacts directly with DNA molecules, disrupting their structures and causing death of cancer cells. However, the majority of radiation-induced damage is caused by an indirect mechanism. Reactive oxygen species (ROS), generated by radiolysis of water, interact with cellular macromolecules such as DNA, RNA, proteins and membranes, leading to cell dysfunction, apoptosis and necrosis (3, 7, 8). Given the direct or indirect damage to the DNA, cells that are highly proliferative (e.g. cancer cells), are particularly radiosensitive (9, 10). Unfortunately, since the radiation field is not limited to tumour tissue, pelvic radiation (PR) therapy also affects healthy, surrounding tissues, such as those of the gastrointestinal (GI) tract. Like cancer cells, the cells lining the GI tract are characterised by a high mitotic rate, making the gut one of the most

sensitive organs to the damaging effects of ionising radiation (11, 12). For this reason, a vast majority of pelvic cancer patients who receive radiotherapy will experience radiation-induced side effects, collectively called pelvic radiation disease (PRD) (13, 14). Patients suffering from PRD can experience a wide range of complications that can be classified into two clinical phases, characterised by a different pathogenesis (15). Within the first three months after PR therapy, the acute phase takes place. Herein, ionising radiation will generate ROS, damaging the cells and ultimately leading to cell death. This causes mucosal breakdown, bacterial translocation and recruitment of immune cells (12, 16). Subsequently, an inflammatory response is initiated, accompanied by increased generation of ROS, as well as pro-inflammatory and profibrotic cytokines, chemokines and growth factors (17, 18). During this acute phase, patients will experience symptoms such as nausea, diarrhoea, fatigue, abdominal pain and bleeding (19, 20). Chronic PRD, the second clinical phase, is a progressive condition that develops between 6 months to several years after PR therapy. Symptoms include intestinal malabsorption, dysmotility and altered transit (19). Chronic PRD can be marked by fibrosis, vasculitis, atrophy and morphological changes in fibroblasts, endothelial and epithelial cells (21). It is important to note that chronic side effects are more likely to develop after severe acute toxicity, as described by the consequential late effect theory (22-24). To this day, treatment interruption or adaptations to the original treatment plan (e.g. reduction in radiation dose and field size) are still the most important measures used in the prevention of PRD (15, 25). However, these methods inevitably decrease treatment efficacy (26). Consequently, research investigating alternative measures to prevent GI complications after PR therapy is warranted.

It has become increasingly evident that the gut microbiome (i.e. the collection of microbes and their genomes present in our digestive system) plays a significant role in our overall health (27). Initial attempts to identify bacteria residing in the gut have been done by culture-dependent techniques. However, the development of new techniques, including metagenomics (i.e. the genomic analysis of microbial communities in environmental samples without need for culturing), has led to more insight into the composition of the gut microbiota (28). It is now known that the predominant phyla residing in the human gut are *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (29-31). In a healthy human being, these communities perform particular functions, such as nutrient metabolism (fermentation and vitamin production), immunomodulation, regulation of gut development, maintenance of epithelial integrity and even regulation of maturation and function of microglia in the central nervous system (32-35).

Certain factors, including the environment, diet and use of medication may induce detrimental shifts in numbers and diversity of microorganisms within a given microbial population, often referred to as dysbiosis (27). This microbial imbalance has been associated with various diseases such as obesity, diabetes, cancer and inflammatory bowel disease (33, 36-39). Recently, studies in both animals and humans have linked gut microbial dysbiosis to the development and progression of PRD, which has been reviewed by Touchefeu et al. (40). Gut microbial dysbiosis contributes to mucositis, diarrhoea, systemic inflammatory responses and fatigue in patients with PRD (41, 42). One study observed dysbiosis among patients with radiation enteritis, characterised by reduced α-diversity (variation within one sample) and increased β-diversity (variation between samples) (43). In patients with radiation enteritis, a higher relative abundance of *Proteobacteria* and *Gammaproteobacteria* and a lower abundance of *Bacteroides* was reported. Additionally, the authors found that patient-derived microbiota induced epithelial inflammation and barrier dysfunction in co-cultures of epithelial cells and bacteria. Other studies showed that the microbial composition of patients who developed diarrhoea after radiotherapy was significantly different prior to radiotherapy (42, 44). In addition, the relative abundance of *Bacteroides* and *Clostridium XIVa* was significantly increased after radiotherapy, while that of *Faecalibacterium*, *Lachnospiracea*, *Oscillibacter*, *Roseburia* and *Streptococcus* was decreased (42). In another study, it was found that when germ-free mice were inoculated with faecal microbiota obtained from irradiated mice and exposed to radiation, mice inoculated with radiation-exposed microbiota showed more severe radiation injury (41). Although not all studies report the same alterations in microbiota, their results

emphasize the importance of prevention of radiation-induced gut microbial dysbiosis and restoration of bacterial homeostasis after PR therapy. Hence, in addition to traditional methods to reduce radiotoxicity, scientists have been exploring adjuvant therapies, including food supplements such as vitamins, prebiotics and probiotics. Probiotics are defined as "live micro-organisms, which, when administered in adequate amounts, confer a health benefit on the host" (45). Positive effects of probiotics on the general intestinal health, including intestinal barrier improvements, inhibition of pathogens, stimulation of beneficial bacteria and reduction of the intestinal inflammatory profile in both animals and humans have been extensively reviewed by Segers et al. (20). Microbial therapies such as VSL#3® and *Lacticaseibacillus* [basonym *Lactobacillus* (46)] *rhamnosus* GG ATCC 53103 have already shown promising results in treating radiotherapy-induced diarrhoea in clinical studies (47, 48). However, most studies have only reported modest effects with no overall improvements in all study participants, suggesting inter-individual variability for probiotics' success. Additionally, underlying protective mechanisms of probiotics against PRD are yet to be described. Thus, further research is needed to investigate the effects of PR therapy on the GI tract as well as the gut microbiome and to identify new food supplements to counteract radiation-induced side effects.

One novel, interesting candidate to combat PRD is the edible photosynthetic cyanobacterium *Limnospira indica* PCC8005. *Limnospira* species were formerly classified in the genus *Arthrospira*, also known as *Spirulina*, which commonly refers to the dried biomass of commercially grown *Arthrospira* species (49). *Limnospira* contains high amounts of proteins (up to 70%), vitamins (e.g. vitamin B12), provitamins (e.g. β-carotene) and minerals. Additionally, it is rich in phenolic acids, tocopherols and γ-linolenic acid (50). Therefore, *Limnospira* has long been used as a food supplement and has been listed by the US Food and Drug Administration in the category Generally Recognized as Safe (GRAS) (51). Aside from its high nutritional content, *Limnospira* has also attracted attention because of its therapeutic effects (50). Many studies have shown that *Limnospira* has a wide variety of health benefits, including immunomodulatory and anticancer activities (52-55). Additionally, *Limnospira* exhibits antioxidant enzyme activity by preventing lipid peroxidation, DNA damage and scavenging free radicals (52). The molecular mechanisms responsible for the effects of *Limnospira* remain unknown. However, (allo-)phycocyanin, β-carotene, tocopherol, γ-linolenic acid and phenolic compounds appear to play an important role (56-59). Besides the above-mentioned effects, it has been shown that *Limnospira* is able to inhibit growth of certain Gram-negative (e.g. *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*) and Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus pumilus*) *in vitro* (60). On the contrary, it can also promote *in vitro* growth of lactic acid bacteria (e.g. *Lactococcus lactis* and *Lacticaseibacillus casei*) and was shown to stimulate growth of *Bifidobacterium*. For this reason, it has been used to enhance viability of bacteria present in fermented milk (61, 62). Recent studies in animal models showed that *Limnospira* could modulate the composition of the gut microbiota. Specifically, one study showed that orally administered *Limnospira* suspension altered the colonic microbiota in a dose-dependent manner in mice (63). At a low dose, the abundance of *Clostridium Cluster XIVa* increased, while at a high dose, the abundance of *Bacteroides* increased. Another study showed that phycocyanin (PC) isolated from *Limnospira* increased bacterial diversity and richness, decreased bacteria related to inflammation and increased short-chain fatty acids (SCFAs)-producing bacteria in mice with bleomycin-induced pulmonary fibrosis (64). Lastly, it was reported that PC ameliorated radiation-induced injury in a mouse study through positive modulation of the gut microbiota composition. Moreover, it was found that these changes lowered lipopolysaccharide levels and suppressed inflammation-inducing TLR/Myd88/ NF-κB activations. Finally, PC also seemed to decrease levels of inflammatory cytokines, such as TNF-α and interleukin-6 (65). Taken together, these characteristics make *Limnospira* a potential candidate to combat the effects of ionising radiation. Another candidate is *Lacticaseibacillus rhamnosus* GG ATCC 53103 (46). *Lacticaseibacillus* species are lactic acid bacteria (LAB) that colonize different sites of the human body (e.g. digestive system, urogenital system) and are among the most commonly used probiotics (66,

67). *L. rhamnosus* in particular is known to modulate the gut microbiota both in animals and humans (68-71). Additionally, it is reported to have anti-inflammatory activities and is even thought to inhibit cancer cell proliferation and tumour invasion (72-75). Besides these benefits, several studies have linked administration of *L. rhamnosus* to a reduction in radiation-induced toxicity (76, 77). This study is part of an ongoing research project investigating the potential use of *Limnospira indica* PCC8005 and *Lacticaseibacillus rhamnosus* GG ATCC 53103 as therapeutic agents to prevent PRD in a mouse model. This particular study aimed to evaluate the effects of PR on intestinal tissues. To investigate this, healthy, non-supplemented C57BL/6J mice were locally irradiated and their intestinal tissues were analysed. Additionally, healthy, non-irradiated mice were supplemented with *Limnospira indica* PCC8005 or *Lacticaseibacillus rhamnosus* GG ATCC 53103. Abdominal tissues were collected and examined for macroscopic changes to assess overall safety of the above-mentioned microbial food supplements.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

The *Limnospira indica* PCC8005 strain used in this study was obtained from the Pasteur Culture collection of Cyanobacteria (PCC) (Institute Pasteur, Paris, France). *Limnospira* cultures were grown axenically in Zarrouk medium (78) at pH ~ 9.8 on a Heidolph Unimax 2010 rotary shaker (Analis SA, Namur, Belgium) at 120 rpm and a constant temperature of 30°C in a Binder KBW400 growth chamber (Analis SA, Namur, Belgium). Cells were illuminated at a photon flux density of 45 µmol photons m^{-2} s^{-1} produced by Osram Daylight tubes (Osram, Zellik, Belgium). Optical density (OD) of the cultures was measured using a Thermo Spectronic Unicam Aquamate Helios Spectrophotometer (Thermofisher Scientific, Merelbeke, Belgium). When an OD of ~ 1.0 was reached at 750 nm, subcultures were made. Contents of several pigments, including chlorophyll, (allo-)phycocyanin [(A)PC] and carotenoids, were determined as described in supplementary methods. On the other hand, *Lacticaseibacillus rhamnosus* GG ATCC 53103 (46), a strain originally isolated from human faecal samples (79, 80), was obtained from Sarah Lebeer (University of Antwerp) and grown statically at 37 °C in de Man, Rogosa and Sharpe (MRS) medium (Difco, Erembodegem, Belgium). To prepare bacteria for supplementation, *Limnospira* was grown until an OD_{750} ~ 1 was reached, at which point 1 L of culture contained ~ 1 mg/mL *Limnospira* bacterial cells. This concentration was determined by measuring dry weight of cultures. Briefly, 1 mL of *Limnospira* was collected on a Supor® 0.2 µm PES filter (Pall Corporation, Hoegaarden, Belgium), which was dried overnight at 60°C. Filters were weighed on their own and after collecting and drying bacterial cells. *L. rhamnosus* was grown until an OD_{600} of ~ 0.25 was reached, corresponding to 10^8 CFU/mL culture. This concentration was determined using flow cytometry. Briefly, bacterial suspensions were diluted in 0.2 µm filtered PBS (1:1,000). Samples were added into a 96-well plate. Next, a 1:100 working solution of SYBR Green I (Invitrogen, Merelbeke, Belgium) was made by diluting the stock solution in dimethyl sulfoxide (DMSO). Lastly, samples were incubated with this solution for 20 min at 37°C in the dark and ran on a flow cytometer to analyse total cell numbers (BD Accuri C6, BD Biosciences, Erembodegem, Belgium). Optical filters were set up such that SYBR Green I could be detected in FL-1 (533±15 nm) with a blue laser (488 nm). Ultimately, cultures were centrifuged at 10,000 x g for 10 minutes at 4°C and washed three times using saline solution. Bacteria were brought up in volumes to reach the desired amount of bacteria per 200 µL for murine gavage.

Animal model

All experiments were approved by the Ethical Animal Welfare Committee of Medanex Clinic, in compliance with the Belgian laboratory animal legislation and the European Communities Council Directive of 22 September 2010 (2010/63/EU) (EC MxCl 2018-093). Five-week-old C57BL/6J male mice were purchased from Janvier (Bio Services, Uden, the Netherlands). All mice were housed individually in ventilated cages and kept under standard laboratory conditions (12 hour light/dark cycle). During acclimatisation, all groups were fed *ad libitum* using regular chow (Carfil Quality, Oud-Turnhout, Belgium). In the first experiment, this diet was followed by diet gels starting seven days before irradiation (DietGel® 76A Bio Services, Uden, the Netherlands) supplemented with 10% maltodextrin (Applichem, Darmstadt, Germany). Maltodextrin is a commonly used placebo in dietary studies evaluating gut microbiota and intestinal

well-being and has no anticipated effects on colonic fermentation (81). In the second experiment, only above-mentioned regular chow was used.

Experimental design

First, to assess the effects of PR on inflammation and functional integrity of gut tissue, healthy, non-supplemented mice were irradiated and intestinal tissues were analysed (Fig. S1A). After 2 weeks of acclimatisation, mice were randomised into 6 different groups (n = 7/group): (1) irradiated mice, sacrificed 1 day after irradiation (PID1); (2) irradiated mice, sacrificed 3 days after irradiation (PID3), (3) irradiated mice, sacrificed 7 days after irradiation (PID7) and time-matched, sham-irradiated controls (4-6). Overall health was monitored, and body weight (BW) and food intake were recorded regularly. Second, to assess the toxicity of bacterial supplementation, non-irradiated mice were supplemented and their organs were collected (Fig. S1B). After 2 weeks of acclimatisation, mice were randomised into 3 different supplementation groups (n = 10/group): (1) controls, receiving saline suspension (200 µL/mouse/day); (2) mice receiving *L. rhamnosus* (10^9 colony forming units or CFU /mouse/day) and (3) mice receiving *Limnospira* (~800 mg/kg BW/day). Suspensions were administered daily by oral gavage in a maximum volume of 10 µL/g BW. Thus, mice of around 20 g received 10^9 CFU of *L. rhamnosus*, solved in 200 µL saline solution, or 16 mg of *Limnospira*, solved in 200 µL saline solution, on a daily basis. The amount of *L. rhamnosus* was expressed in CFU as it is an estimate of the number of viable bacteria of a culture. The administered amount, 10^9 CFU/mouse/day, was chosen because it is a sufficient quantity to produce beneficial effects (82). The amount of *Limnospira* administered to mice was expressed in mg/kg BW, as, in contrast to *L. rhamnosus*, *Limnospira* does not form individual colonies and thus cannot be expressed in CFUs. In addition, this expression allows for calculation of administered bioactive compounds, such as (A)PC, chlorophyll and carotenoids. After two weeks of supplementation, an additional two-week wash-out period was introduced. Overall health was monitored, and BW was recorded regularly. At the end of the wash-out period, all mice were sacrificed to assess spleen size and weight, colon length and caecum weight.

Irradiation protocol

Fifteen minutes before irradiation, mice were administered a combination of 50 mg/kg ketamine hydrochloride (Nimatek, Eurovet, Bladel, the Netherlands) and 0.25 mg/kg medetomidine hydrochloride (Domitor, Elanco, Antwerp, Belgium) by means of an intraperitoneal injection. An eye gel comprising 2 mg/g carbomerum (Vidisic, Bausch+Lomb, Brussels, Belgium) was used to avoid dry eyes during the anaesthetic procedure. During irradiation, mice were placed in prone position in a Plexiglas pie cage (20 cm diameter, 5 cm height), with their lower body parts towards the center of the cage. Apart from the center, the cage was covered by a 5 mm thick lead shield to ensure local, pelvic irradiation of the mice (Fig. S2). This pie structure was placed on a heating plate (35-37°C) to allow mice to maintain their body temperature. Acute, single-dose X-irradiation of 12 Gy was carried out using an Xstrahl device, which operated at 320 kV, 12 mA and a dose rate of 78 cGy/min. After ca. 17 minutes of irradiation, anaesthesia was reversed using 1 mg/kg atipamezole hydrochloride (Antisedan, Elanco, Antwerp, Belgium).

Tissue collection

In both experiments, mice were sacrificed using 50 mg/kg ketamine hydrochloride (Nimatek, Eurovet, Bladel, the Netherlands) and 0.25 mg/kg medetomidine hydrochloride (Domitor, Elanco, Antwerp, Belgium) by means of an intraperitoneal injection. In the irradiation experiment, spleen, jejunum, ileum and colon were excised and surrounding fat tissue was removed. Blood was collected by cardiac puncture. Tissue samples were measured and/or weighed, snap frozen and stored at -80°C. In the supplementation experiment, spleen, colon and caecum were collected, measured and/or weighed. Additionally, the caecum was weighed and 50 mg caecal content was snap frozen and stored at -80°C.

Intestinal permeability assay

Intestinal permeability was assessed by means of the fluorescein isothiocyanate (FITC)-dextran assay. After overnight starvation, FITC-dextran (FD4, 3-5 kDa, Sigma, Overijse, Belgium) dissolved in phosphate-buffered saline (PBS) was administered to each mouse by oral gavage (44 mg/100 g BW). After four hours, mice were sacrificed and blood was collected by cardiac puncture and transferred to capillary blood tubes (Sarstedt, Nümbrecht, Germany). Once blood was collected, tubes were centrifuged (10,000 x g, 5

min) (Centrifuge 5418 R, Eppendorf, Aarschot, Belgium) to separate the serum. Afterwards, the serum was diluted with an equal volume of PBS and 100 µL of diluted serum was added to a 96-well microplate (Greiner, Vilvoorde, Belgium). Fluorescence was measured using a CLARIOstar microplate reader (excitation 483 nm, emission 530 nm) (BMG Labtech, Isogen LifeScience, Utrecht, the Netherlands). FITC-dextran concentration in serum was determined using standard curves of serially diluted FITC-dextran. Serum from mice not administered with FITC-dextran was used to determine the background signal.

MPO activity assay

Activity of myeloperoxidase (MPO), a pro-inflammatory enzyme stored in granules of neutrophilic granulocytes, was determined in jejunum, ileum and colon tissue (83). All procedures were performed on ice. Samples were suspended in buffer at a ratio of 5 g tissue/100 mL buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM potassium hydrogen phosphate, pH 6) and homogenized using a Qiagen TissueLyser (2 x 20 s, 25 Hz) (Qiagen, Venlo, the Netherlands). Next, the suspension was centrifuged at 14,000 x g for 15 minutes at 4°C (Centrifuge 5418 R, Eppendorf, Aarschot, Belgium) and supernatant was stored at -80°C. Afterwards, in a 96-well plate, 290 µL of assay reagent (100 µL 16.7 mg/mL O-dianisidine dihydrochloride in 0.9% sodium chloride, 100 µL 0.05% H_2O_2, 9.8 mL 50 mM potassium hydrogen phosphate) was added to 10 µL of supernatant to start the reaction. Absorbance (460 nm) was measured at 0 and 60 seconds using a CLARIOstar microplate reader (BMG Labtech, Isogen LifeScience, Utrecht, the Netherlands). Measurements were performed in triplicate. One unit of MPO was defined as the amount needed to convert 1 µmol of H_2O_2 into H_2O per minute at 25°C. The activity was expressed in units (U) MPO per milligram of tissue and was calculated as followed:

$$\text{U MPO/mg tissue} = [A_{460}\ (60s) - A_{460}\ (0s)] * 0.273 * 200$$

where A_{460} is the absorbance measured at 460 nm.

Bacterial translocation assay

Bacterial translocation (BT), i.e. migration of viable bacteria from the intestinal lumen to mesenteric lymph nodes (MLN), was determined by flow cytometry. Briefly, MLNs, which receive lymph coming from the distal ileum and proximal colon, were collected aseptically and transferred to cold PBS. Next, they were mechanically homogenized through a 40 µm nylon cell strainer using a 10 mL syringe plunger before analysis by flow cytometry. Briefly, samples were diluted in 0.2 µm filtered PBS (1:5,000) to obtain an event rate between 200 and 2,000 events/µL, which allows for more accurate measurements. Next, samples were filtered using a 5 µm syringe filter to remove eukaryotic cells. Samples were added into a 96-well plate. Next, a 1:100 working solution of SYBR Green I (Invitrogen, Merelbeke, Belgium) was made by diluting the stock solution in dimethyl sulfoxide (DMSO). Lastly, samples were incubated for 20 min at 37°C in the dark and ran on a flow cytometer to analyse total cell numbers (BD Accuri C6, BD Biosciences, Erembodegem, Belgium). Optical filters were set up such that SYBR Green I could be detected in FL-1 (533±15 nm) with a blue laser (488nm).

Statistical analyses

Data from BW changes and food intake are presented as mean ± standard error of mean. Linear regression models were fitted to the data to describe the relationship between different variables. Next, data were analyzed using one-way analysis of variance (ANOVA) with Tukey's post-hoc test in R (version 3.6.2). Outliers were detected based on Tukey's fences method (values below Q_1 - 1.5 IQR or above Q_3 + 1.5 IQR) and excluded from statistical analysis (84). Statistical significance was considered at $P < 0.05$.

RESULTS

Pelvic radiation does not affect body weight and food intake

BW and food intake were determined as a measure for overall health. BW was expressed as percentage of initial BW and measured up to seven days after (sham-) irradiation. Cumulative food intake was determined by weighing diet gels six days before and after (sham-)irradiation. At baseline, no significant differences in BW were noted between control mice and irradiated mice. After one day, BW of all mice decreased, after which they increased again and remained stable up to six days after irradiation. On the seventh day, all mice had lost weight due to fasting procedures needed for the FITC-dextran assay prior to sacrifice. No statistical differences were found between controls and

irradiated mice over the course of seven days (Fig. 1A). Food intake at baseline did not differ between controls and irradiated mice. In addition, no differences were found between the two groups after (sham-)irradiation (Fig. 1B). Taken together, these results indicate that PR does not affect body weight, nor food intake.

Pelvic radiation reduces colon and spleen size

Colon length, which is inversely related to intestinal inflammation, was measured from anus until caecum. Although a linear regression model showed no overall effect of dose or time on colon length, colon length of irradiated mice was significantly decreased when compared to control mice three days after irradiation ($P = 0.018$). Seven days post-irradiation, this difference was no longer observed (Fig. 2A), suggesting that PR induces apoptosis and short term inflammation, which leads to a temporal reduction in colon length at PID3, after which colon length is restored. Additionally, spleen size was measured. Overall, a significant inverse relationship was detected between dose and spleen size ($P < 0.001$), as well as time and spleen size ($P = 0.016$). Moreover, a significant interaction effect was noted between time and dose ($P = 0.003$), suggesting that with increasing time, spleen size differences between control and irradiated mice decreased. Spleens were significantly smaller in irradiated mice than in control mice both one and three days after irradiation ($P = 0.038$ and $P = 0.015$, respectively). This reduction was no longer observed at seven days post-irradiation (Fig. 2B), suggesting that PR induces a transient reduction in spleen size, after which spleen size is restored.

Pelvic radiation induces inflammation and bacterial translocation while changes in intestinal permeability could not be detected

To assess intestinal inflammation and infiltration of neutrophils, MPO activity was determined in the jejunum, ileum and colon of mice. In jejunal tissue, a linear regression model showed an overall increase of MPO activity in irradiated mice ($P = 0.013$). In addition, a significant interaction effect was found between time and dose ($P = 0.005$), indicating that as time and dose increased, jejunal MPO activity decreased. When looking at different time points, no significant difference was found at PID1, nor at PID3. However, on the seventh day after irradiation, MPO activity in the jejunum of irradiated mice was significantly lower than in control mice ($P = 0.020$) (Fig. 3A). In the ileum of irradiated mice, the linear regression model showed that both time and dose significantly increased MPO activity ($P = 0.033$ and $P = 0.049$, respectively). Specifically, ileal MPO activity was found to be significantly increased one day after radiation ($P = 0.015$), but not at PID3 and PID7 (Fig. 3B). In the colon, the linear regression model indicated that neither time nor dose significantly altered MPO activity. Consequently, no differences were seen between control and irradiated mice at the different time points (Fig. 3C). Taken together, these results suggest that, upon PR, an inflammatory response is initiated in the jejunum and ileum. In jejunal tissues, this subsides over the course of seven days, while the increasing trend is maintained over time in ileal tissues. Besides MPO activity, the concentration of FITC-dextran was

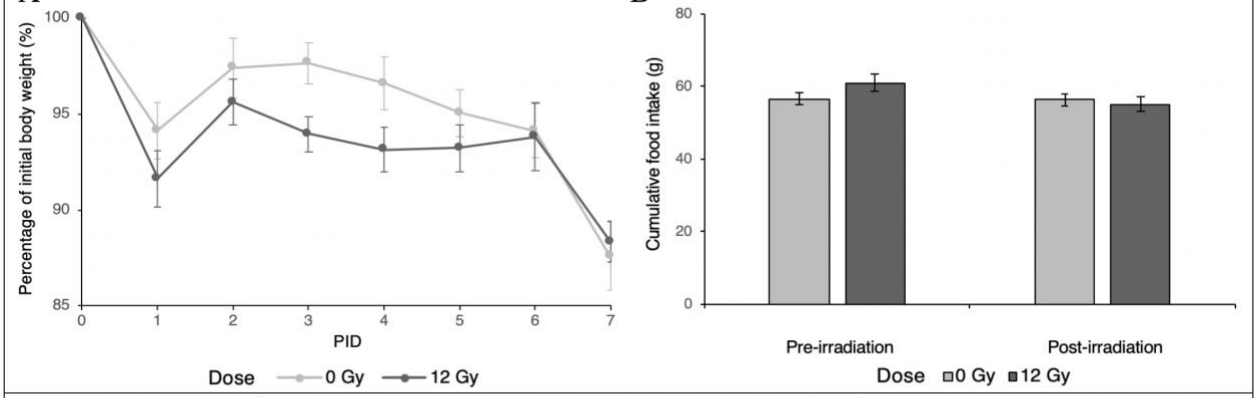

Fig. 1 – Body weight (BW) and food intake following pelvic radiation. (A) BW is not affected by pelvic radiation. (B) Cumulative food intake is not altered following pelvic radiation. Food intake was measured six days before/after (sham-)irradiation. Linear models were created and comparisons were made using one-way ANOVA and Tukey's post-hoc testing in R. All data represent mean values ± SEM. $n_{control\ mice} = 6$, $n_{irradiated\ mice} = 7$. PID = post-irradiation day.

measured in the serum of mice, as a marker of intestinal permeability. A linear regression model showed that overall, dose, as well as time significantly decreased the concentrations of FITC-dextran in mice (P = 0.020 and P = 0.010, respectively). However, no significant differences were found when comparing control and irradiated mice at different time points (Fig. 3D). This shows that the FITC-dextran assay did not put forward any radiation-induced changes in intestinal permeability. Lastly, the occurrence of BT from the distal ileum and proximal colon to MLNs was investigated. When looking at BT, flow cytometry counts were significantly lower in MLNs of irradiated mice than those of control mice at PID1 and 3 (P = 0.009 and P = 0.013, respectively). However, at PID7, counts were significantly higher in irradiated mice compared to their time-matched controls (P = 0.022) (Fig. 3E). This suggests that while PR initially decreases the occurrence of BT, eventually, an increase will occur in the amount of bacteria translocating to the MLNs.

Analysis of *Limnospira* pigments and proteins

Limnospira content was analyzed using aliquots originating from one culture (OD ~ 1). This culture contained 0.15 mg/mL PC, 0.06 mg/mL APC, 0.03 mg/mL chlorophyll and 1.85 mg/mL carotenoids. Total protein concentration was 1.06 mg/mL *Limnospira* culture.

Relative body weight is overall lower in *Limnospira* supplemented mice

Relative BW, expressed as percentage of initial BW, was determined as a measure for overall health and measured from the start of supplementation until the end of the wash-out period. At baseline, no significant differences in BW were noted between saline and *Limnospira* and *L. rhamnosus* groups. A linear regression model showed that overall, the relative BW in the *Limnospira* supplemented group was lower than in the saline group (P = 0.004). An overall effect of time was also noted. As time increased, BW increased (P < 0.001). In addition, as time increased, the effect of *Limnospira* supplementation decreased (P = 0.006). *L. rhamnosus* supplementation did not affect BW (Fig. 4). These results indicate that relative body weight is overall lower in *Limnospira* supplemented mice, and not altered in *L. rhamnosus* supplemented mice.

Caecum weight is not altered following supplementation

Caecum weight, as a measure for bacterial load in the caecum, was assessed. The linear regression model showed that caecum weight was unaltered by *Limnospira* and *L. rhamnosus* supplementation. Consequently, no significant differences could be detected when comparing the three groups (Fig. 5A). These results show that caecum weight is not affected by supplementation.

***Limnospira* and *L. rhamnosus* do not alter colon length, but increase spleen size**

Colon length was measured at the end of the wash-

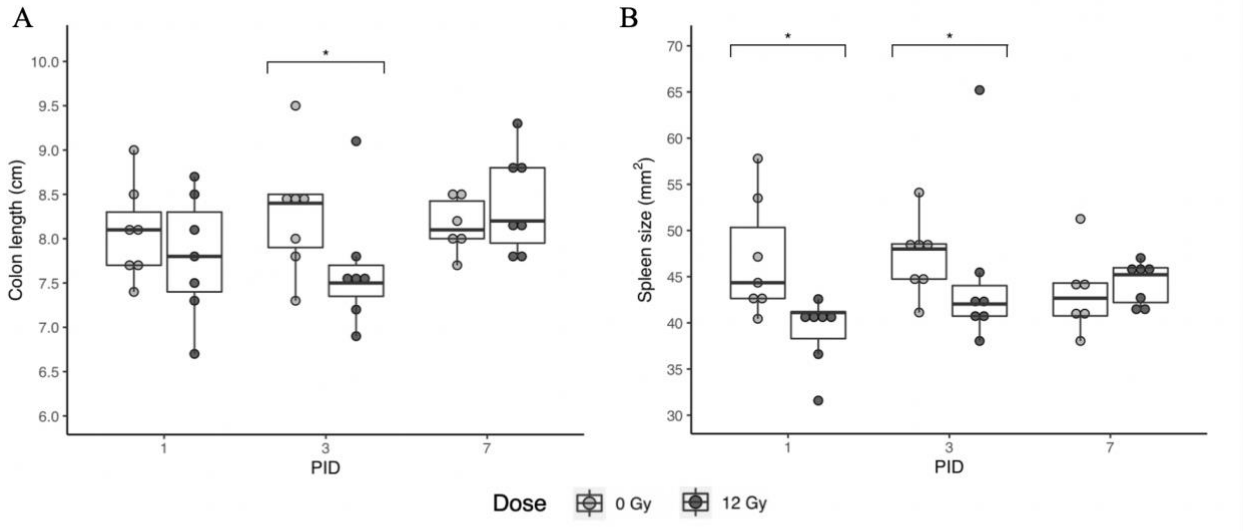

Fig. 2 – Colon length and spleen size following pelvic radiation. (A) Colon length (cm) and (B) spleen size (mm^2) are reduced by pelvic radiation. Linear models were created and comparisons between groups were made by one-way ANOVA and Tukey's post-hoc testing in R. Outliers were excluded from statistical analysis (Tukey's method). $n_{control\ mice}$ = 6-7, $n_{irradiated\ mice}$ = 7. *P < 0.05. PID = post-irradiation day.

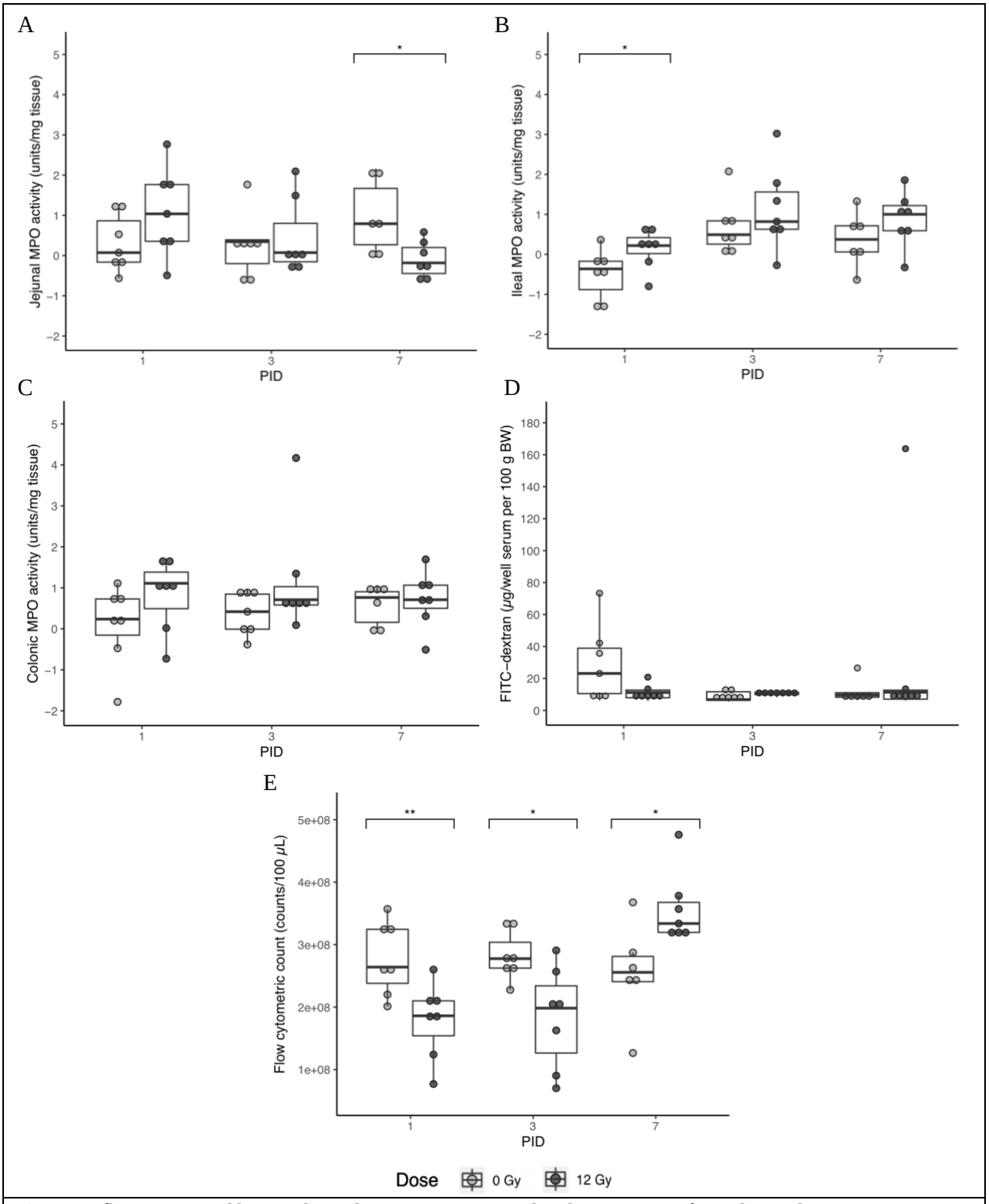

Fig. 3 – Inflammation and bacterial translocation are increased in the intestines after pelvic radiation. MPO activity (units/mg tissue) in (A) jejunum, (B) ileum and (C) colon was determined as a measure for intestinal inflammation. (D) Intestinal permeability was measured by means of FITC-dextran concentration (µg/well serum/100 g BW) in serum of mice. (E) Bacterial translocation was assessed by flow cytometric counting (counts/100 µL). Linear models were created and comparisons were made by one-way ANOVA and Tukey's post-hoc testing in R. Outliers were excluded from statistical analysis (Tukey's method). $n_{control\ mice}$ = 6-7, $n_{irradiated\ mice}$ = 7. **P* < 0.05, ***P* < 0.01. PID = post-irradiation day, MPO = myeloperoxidase, FITC = fluorescein isothiocyanate.

out period and appeared not to be affected by either of the supplementation regimens (Fig. 5B). In addition, both spleen size and weight were assessed. The linear regression model showed an overall increase in spleen size after *Limnospira* supplementation ($P = 0.048$) (Fig. 5C). Differences in spleen weight could not be detected (Fig. 5D). These results show that none of the supplementation strategies altered colon length. In addition, spleen size is only significantly increased by *Limnospira* supplementation.

DISCUSSION

The present study evaluated the effects of PR on murine intestinal tissues and assessed the safety of two potential mitigators of PRD, *Limnospira indica* PCC8005 and *Lacticaseibacillus rhamnosus* GG ATCC 53103. First, the effects of PR on body weight and food intake were investigated. No significant differences were found in BW of control and irradiated mice up until seven days. In addition, no differences in food intake were found. These results indicate that PR neither affects BW, nor food intake. This is in contrast to other studies that showed weight loss and mortality after abdominal irradiation. For example, in two different studies, Jia et al. observed dose-dependent weight loss in C57BL/6 mice exposed to 5 to 20 Gy of abdominal radiation (85, 86). In both studies, body weight loss started as early as one day after irradiation, regardless of the radiation dose. Weight loss in mice peaked on day three to five, after which surviving mice gradually regained their weight. While in both studies, the mouse strain and dose are comparable, dose rates used in our experiment were lower, and could explain the discrepancy in observations (87). In addition, the irradiated field plays an important role in the observed effects post-irradiation. On this note, PR can result in radiation-induced gastro-intestinal syndrome, while total-body irradiation (TBI) can also induce hematopoietic syndrome, characterized by a reduction in blood cells and associated with more severe radiation-induced adverse outcomes, including more pronounced weight loss (88). Besides BW and food intake, macroscopic effects of PR on colon and spleen were assessed. Radiation-induced intestinal inflammation can be characterised by thickening and shortening of the colon (89, 90). In this study, colon length was significantly reduced in irradiated mice at PID3. This reduction in colon length could be an indicator that after PR, apoptosis and inflammation occur in the colon of mice, after which the cells in the colon regenerate (91, 92). Previous (unpublished) data from our research group have shown apoptosis in the colon one day post-irradiation, supporting the above-mentioned hypothesis. The subsequent rapid recovery can be explained by the high turn-over rate of the intestinal tissue, which makes regeneration possible even if only a limited number of stem cells in the intestinal crypts would have survived (93, 94). Besides a reduction in colon length, a reduction in spleen size in irradiated mice was detected. Overall, there was an inverse relationship between

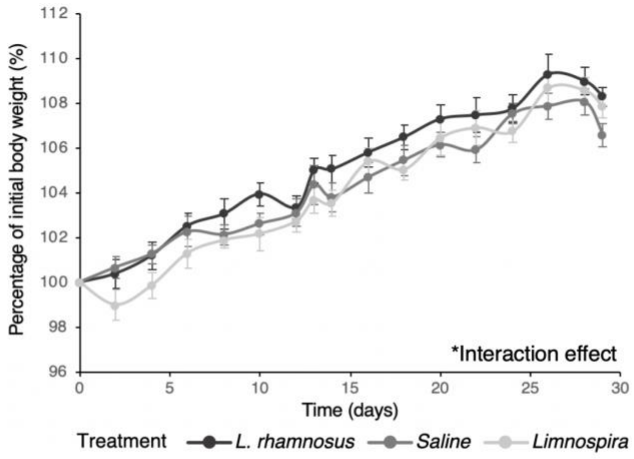

Fig. 4 – Relative body weight following supplementation. Overall, relative body weight is lower in *Limnospira* supplemented mice than in controls ($P < 0.01$), although there are no significant differences at specific time points. A significant interaction effect is noted between time and treatment. Linear models were created and comparisons were made by one-way ANOVA and Tukey's post-hoc testing in R. All data represent mean values ± SEM. $n_{saline} = 10$, $n_{Limnospira} = 10$, $n_{L.\ rhamnosus} = 10$.

dose and spleen size, suggesting that PR induces a decrease in spleen size. However, the reduction in spleen size at PID3 could no longer be detected at PID7, suggesting regeneration of the spleen tissue. It has previously been reported that radiation induces reduction in spleen weight and size in mice. In line with our results, Kachikwu et al. found that low doses (0.25-2 Gy) of TBI reduced spleen size, weight and splenocyte numbers in C57BL/6 mice (95). In addition, Pecaut et al. observed a dose-dependent loss of spleen mass in C57BL/6 mice four days after 0.5 to 3 Gy (high and low dose rate) of TBI (96). The mouse spleen contains areas of myeloid activity, in which stem cells are found, making this organ susceptible to the damaging effects of ionising radiation (97). Consequently, a radiation-induced decrease in spleen size and/or weight has been linked to apoptosis of splenocytes. Takahashi et al. observed a dose-dependent accumulation of apoptosis-associated p53 and Bax proteins and induction of apoptosis in the spleen 12 h after 1.5-4.5 Gy of TBI in C57BL/6N mice. In mice receiving 3 Gy of TBI, apoptosis reached its maximum at 12 h PID and rapidly declined between one and two days after radiation (98). Similarly, Staudacher et al. found an increase in apoptosis after 1 Gy of TBI in pKZ1 mice, an outbred strain of C57BL/6J mice. The level of apoptosis was maximal seven hours after irradiation and remained elevated at PID1, before returning to baseline levels (99). Lastly, Lee et al. found an increase in apoptosis in spleens of C57BL/6 mice one and three

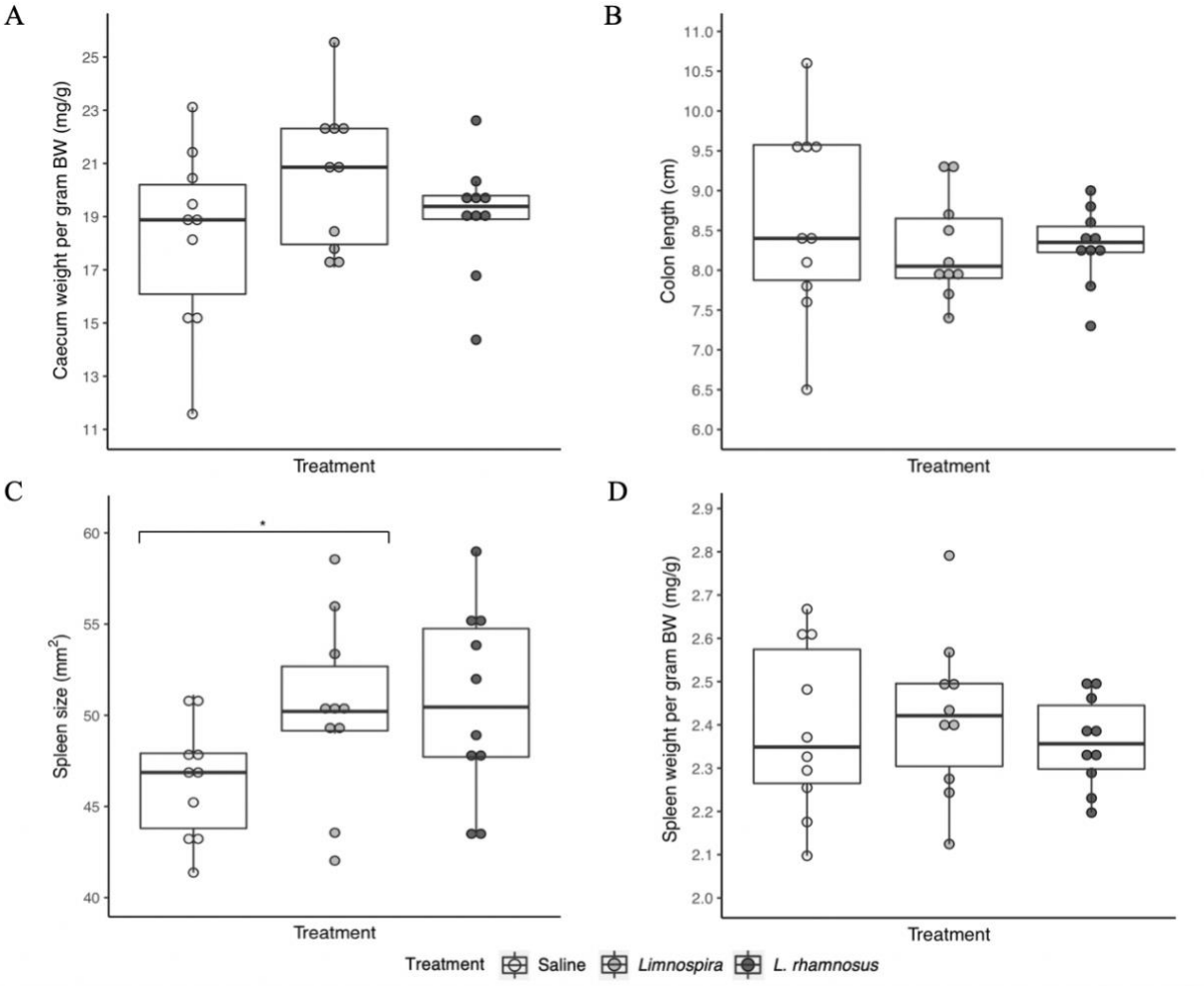

Fig. 5 – Size and weight of different abdominal organs after supplementation. (A) Caecum weight (mg/g BW). (B) Colon length (cm). (C) Spleen size (mm²). (D) Spleen weight (mg/g BW). Organ weights were corrected for body weight (BW). Linear models were created and comparisons between groups were made by one-way ANOVA and Tukey's post-hoc testing in R. Outliers were excluded from statistical analysis (Tukey's method). $n_{saline} = 10$, $n_{Limnospira} = 10$, $n_{L.rhamnosus} = 10$. $*P < 0.05$

days after 0.2 Gy of TBI (100). These studies, in combination with our own observations, suggest that after PR, apoptosis is induced in the spleen, which leads to a reduction in spleen size. This radiation-induced apoptosis subsides after only a few days, allowing for regeneration of spleen cells, and normalisation of the spleen size.

In addition to macroscopic outcomes, effects of PR on inflammation, BT and intestinal permeability were determined. In this study, we measured MPO activity as a marker for inflammation. We showed that upon PR, a rapid, transient inflammatory response, characterised by an increase in MPO activity, is initiated in jejunum and ileum. This is in accordance with findings in literature. For example, Guney et al. found an increase in MPO activity in the ileum of Wistar rats 48 h after 8 Gy of PR (101). Similar to our observations, Freeman et al. did not detect an increase in MPO activity in the colon of C57BL/6 mice, three days after 10 Gy TBI (102). Generally, these findings support the hypothesis that upon ionizing radiation, cytokines and chemokines are released, triggering inflammation and recruitment of neutrophils and other immune cells. In contrast to our study, Deniz et al. observed a radiation-induced increase in MPO activity in the ileum and colon of Sprague-Dawley rats four days after 11 Gy of TBI (103). In addition, increased MPO activity in the colon was also detected by Mihaescu et al., who observed increased levels of MPO in the colon of C57BL/6J mice 16 h after 20 Gy TBI (104). However, the above-mentioned studies adopted a different dose, dose-rate, radiation field and/or animal model. Subsequently, it is possible that in our experiments, the used dose was too low and the irradiated field too small to induce elevated MPO levels in the colon, a part of the GI tract that is reportedly less radiosensitive than the small intestine (105, 106). Future research will have to further elucidate the effects of PR on inflammation in intestinal tissues. This can be achieved by assessing the presence of inflammation-related cytokines, chemokines and growth factors (e.g. IL-1, IL-6, IL-8, TNF-α, IFN-γ) through gene expression analyses or enzyme-linked immunosorbent assays (ELISA) (107, 108).

Besides inflammation, we assessed the effects of PR on intestinal permeability. In this study, no differences in intestinal permeability could be detected between control and irradiated mice. This is in contrast to other reports, stating that intestinal permeability is increased after irradiation. For example, Cui et al. found an increase in FITC-dextran concentrations in C57BL/6J mice 21 days after exposure to 6.5 Gy TBI (109). In addition, Shukla et al. reported disruption of epithelial tight junctions and an increase in FITC-inulin concentration in the blood of C57BL/6 mice two hours after exposure to 4 Gy of TBI (110). Besides an increase in BT seven days after irradiation, Biju et al. also reported an increase in FITC-dextran concentrations in C57BL/6 mice four days after 10 and 12 Gy of TBI (111). Lastly, Jang et al. found elevated levels of FITC-dextran concentration three and six days, and increased BT six days after exposure to 13.5 Gy of PR in C57BL/6 mice. The latter two also suggest a link between tissue damage, increased intestinal permeability and increased occurrence of BT. Given these findings, we suggest that in our study, the FITC-dextran assay could not detect any differences in intestinal permeability, even though they could have been present. Consequently, it is important to note that while FITC-dextran can be a valuable marker of intestinal permeability, there are many pitfalls associated with the use of markers for intestinal permeability. These include factors that might affect absorption, metabolism and excretion of the used markers, such as individual differences in GI motility, intestinal cell surface, renal function, gastric dilution and diet (112-114). Therefore, it is advised to use a combination of multiple intestinal permeability markers before drawing conclusions. Examples include the use of lactulose/mannitol, polyethylene glycols, ovalbumin, but also the evaluation of tight junction proteins through western blotting or quantitative PCR (112-114).

In this study, a decrease in BT was observed in irradiated mice compared to control mice at PID1 and 3. However, at PID7, a significant increase in bacteria was found in the MLNs of irradiated mice. Similar to our observations, Biju et al. observed an increment in BT in C57BL/6 mice from seven days after 9 and 10 Gy of TBI (111). In addition, a study by Carter et al. did not show increased BT in C57BL/6 mice exposed to 5.5 Gy of TBI up to 72 h after radiation. However, the authors did not evaluate BT at a later time point (115). In contrast to our results, Guzman-Stein et al. found increased BT in Sprague-Dawley rats after 1.1 Gy of PR as early as 12 h post-irradiation (116). Taken together, these studies confirm that ionizing radiation is

capable of inducing BT. Several factors can trigger BT, including disturbances in the intestinal flora, increased mucosal barrier permeability and an impaired immune system (115, 117, 118). All of these factors will enhance intestinal permeability or make it easier for bacteria to cross the GI barrier, allowing for BT to other parts of the body (3, 43, 119, 120). Microbicidal properties of ROS produced by neutrophils are essential in the management of GI infections and inflammation following BT (121). In relation to this, in our study, we observed a delay between radiation and the occurrence of BT in irradiated mice. At PID1 and 3, BT was lower in irradiated mice than in control mice. Thus, although radiation can induce BT eventually, it might first reduce the amount of bacteria present in the gut. Since bacterial cells are more radiation-resistant than eukaryotic cells, this is more likely to happen through an indirect mechanism, namely infiltration of neutrophils and increased activity of MPO (122-124). Consequently, MPO can convert more hydrogen peroxide into hypochlorous acid, a highly reactive species with potent microbicidal and cytotoxic properties (125). Then, as the amount of neutrophils in the intestine declines, more bacteria are able to survive and translocate to MLNs. In parallel, extensive MPO activity accompanied with ROS may increase intestinal tissue damage, eventually allowing for bacteria to translocate to MLNs (117, 126). This hypothesis is supported by results of the MPO assay in our study, which show a rapid but transient increase in MPO activity in the jejunum and ileum after irradiation.

Taken together, our results suggest that while PR only modestly affects BW, it induces a transient inflammatory response, associated with temporarily increased MPO activity in jejunum and ileum and a temporary reduction in colon length. Additionally, while we could not detect changes in intestinal permeability, we found that PR results in delayed BT to MLNs. Lastly, it is suggested that following PR, the spleen suffers collateral damage, associated with a transitory reduction in spleen size.

Our final experiment included supplementation of healthy, non-irradiated mice with *Limnospira* or *L. rhamnosus* to assess possible side effects of these potentially radioprotective microbial agents.

First, BW of mice was determined. At baseline, BW did not differ between saline, *Limnospira* and *L. rhamnosus* groups. We found an overall lower relative BW of *Limnospira* supplemented mice when compared to controls, showing an increase with time. In addition, no significant differences in BW were found when comparing groups at specific time points, suggesting that *Limnospira* only had a mild overall effect. The reason for the initial decrease in relative BW remains unknown, but is most likely not attributed to the gavage technique, since all mice underwent the same procedures. Besides, the effects of repeated oral gavage on weight and overall health of mice are generally thought to be minimal (127, 128). Other reports suggest that neither *Limnospira* or related strains, nor *L. rhamnosus*, induce significant body weight changes in healthy mice. For example, Hutadilok-Towatana et al. observed that daily oral administration of S. platensis (30 g/kg fresh or 10 g/kg dried algae) to Swiss mice showed no effect on body weight over the course of 12 weeks (129). Lastly, two other studies reported no differences between *L. rhamnosus*- treated (1 x 10^8 CFU per mouse, or 5 x 10^7 CFU/g BW daily) and control C57BL/6 mice receiving a standard diet over the course of respectively 13 and eight weeks (130, 131).

Besides assessing BW changes, intestinal tissues were collected and examined for macroscopic changes. In this study, caecum weight remained unchanged by both *Limnospira* and *L. rhamnosus*. In literature, increased caecum weight has been linked with altered bacterial load as a result of pre- or probiotic intake (132, 133). In our study, this has not been confirmed.

We found that neither *Limnospira*, nor *L. rhamnosus* supplementation affected colon length in healthy mice. However, several reports have linked both *Limnospira* and *L. rhamnosus* to protection of the colon from inflammation and associated preservation of colon length in inflammatory diseases (134-136). Therefore, these bacteria might also play a protective role in radiation-induced inflammation and associated shortening of the colon.

Lastly, we found an overall increase in spleen size after *Limnospira* supplementation. This is in accordance with findings in literature. For instance, Hayashi et al. observed an increase in mitogen-induced proliferation of spleen cells coming from mice fed a diet containing 10 and 20% of *S. platensis* over the course of 10 weeks. In addition, the authors found that a hot water extract of *S.*

platensis to an *in vitro* culture of spleen cells also increased proliferation and enhanced interleukin-1 (IL-1) production from peritoneal macrophages (137). In addition, certain components of *Limnospira* are linked to increased spleen cell proliferation. For example, Okai et al. found that several carotenoids caused significant stimulatory effects on the proliferative response of spleen cells derived from BALB/c mice. In addition, the authors observed enhanced release of IL-1α and tumour necrosis factor (TNF-α) from murine peritoneal adherent cells (138). These findings suggest that *Limnospira* induces proliferation in the spleen, along with an upregulation of the immune system. Although we found that *Limnospira* induces an increase in spleen size, a comparable increase in spleen weight could not be detected. Future research should confirm whether these bacteria induce an increase in spleen weight, preferably together with analysis of markers of proliferation and inflammation.

Taken together, these results suggest that neither *Limnospira*, nor *L. rhamnosus* induce any adverse effects or acute toxicity in mice when administered at relatively high doses (800 mg/kg and 10^9 CFU per mouse per day, respectively).

Lastly, the pigment content of *Limnospira* was analyzed. We found substantial amounts of PC, APC, chlorophyll and carotenoids (139, 140). These are pigments that are thought to play an important role in the antioxidant and immunomodulatory activities of *Limnospira* (56-59). It is important to note that pigment content can vary among strains and with use of different culture conditions (139-141). Based on PC and carotenoid concentration of cultures and the used volumes of culture per mouse per day (16 mL per mouse of 20 g per day), we determined the amount of PC and carotenoids given to the mice in our supplementation experiment. These concentrations, 120 mg/kg BW/day, and 1.5 kg/kg BW/day are respectively 2.4 and 7.5 times higher than the amount of PC and carotenoids used in studies that have already demonstrated positive effects of these pigments (64, 65, 142). Nonetheless, no toxicity was detected in the present study.

Tremendous progress has been made to elucidate the potential role of food supplements as mitigators of radiation-induced dysbiosis and damage. However, more research is necessary to further clarify the potential radioprotective capacities of *Limnospira* and *L. rhamnosus*. Here, we discuss some future perspectives. First, *Limnospira* and *L. rhamnosus* have already been shown to alter the gut microbiome (63-65, 68, 69). During the supplementation experiment, faeces were collected during supplementation and wash-out periods. The next step is to use these faeces to perform 16S rRNA gene sequencing and analysis. This is a method to identify and classify bacteria based on polymorphisms in the hypervariable regions of the 16S rRNA gene. This gene encodes 16S rRNA, a component of the 30S small subunit of prokaryotic ribosomes and is present in all bacteria. Isolated bacterial DNA can be identified by comparing the 16S rRNA gene sequence to sequences saved in databases (143, 144). This way, it will be possible to determine how supplementation affects the murine gut microbiome. In addition, the minimal duration of supplementation regimens needed to establish these microbial changes will be assessed and it will be determined how long these established microbial alterations last.

Another interesting question to be addressed is how *Limnospira* and *L. rhamnosus* could affect the production of SCFAs. These are released through fermentation of fiber and resistant starches by certain bacteria, and are suggested to exert anti-inflammatory and antimicrobial activities (145, 146). Xie et al. showed that PC isolated from *Limnospira* increased bacterial diversity and richness, decreased bacteria related to inflammation and increased SCFA-producing bacteria in mice with bleomycin-induced pulmonary fibrosis (64). Another study by Neyrinck et al. found an increase in *Roseburia* bacteria, a SCFA producing genus, after supplementing mice with a 5% *S. platensis* diet (147, 148). Consequently, it is suggested that *Limnospira* could play a protective role in reducing radiation-induced damage and dysbiosis through stimulation of SCFA production.

Lastly, the effects that are exerted when both bacteria are combined in one supplementation regimen remain largely unknown. Ghazy et al. have already reported a synergistic ameliorative effect of *Lacticaseibacillus* and *S. platensis* in colitis in rats (149). However, it would be interesting to test such supplementation strategies in light of PRD.

We conclude that pelvic radiation induces inflammation in GI tissues, atrophy in extraintestinal organs such as the spleen and

bacterial translocation to MLNs. In addition, supplementation of food supplements to healthy mice is unlikely to cause adverse effects on different organs in the abdominal cavity. Lastly, *Limnospira* induces an increase in spleen size in mice, indicating its potential immunomodulating activities. Therefore, we suggest that the use of food supplements, namely *Limnospira indica* PCC8005 and *Lacticaseibacillus rhamnosus* GG ATCC 53103, provides a safe and promising approach to reduce radiotherapy-induced GI complications, which might lead to enhanced therapy compliance, treatment outcome and quality of life in patients undergoing pelvic radiotherapy.

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AUTHOR CONTRIBUTIONS

CS, MV and FM designed the experiments. EH and CS performed the experiments. EH and CS analyzed the data. EH wrote this paper with support from CS, MV, FM and MP.

DISCLAIMER

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SUPPLEMENTARY MATERIAL
Supplementary figures

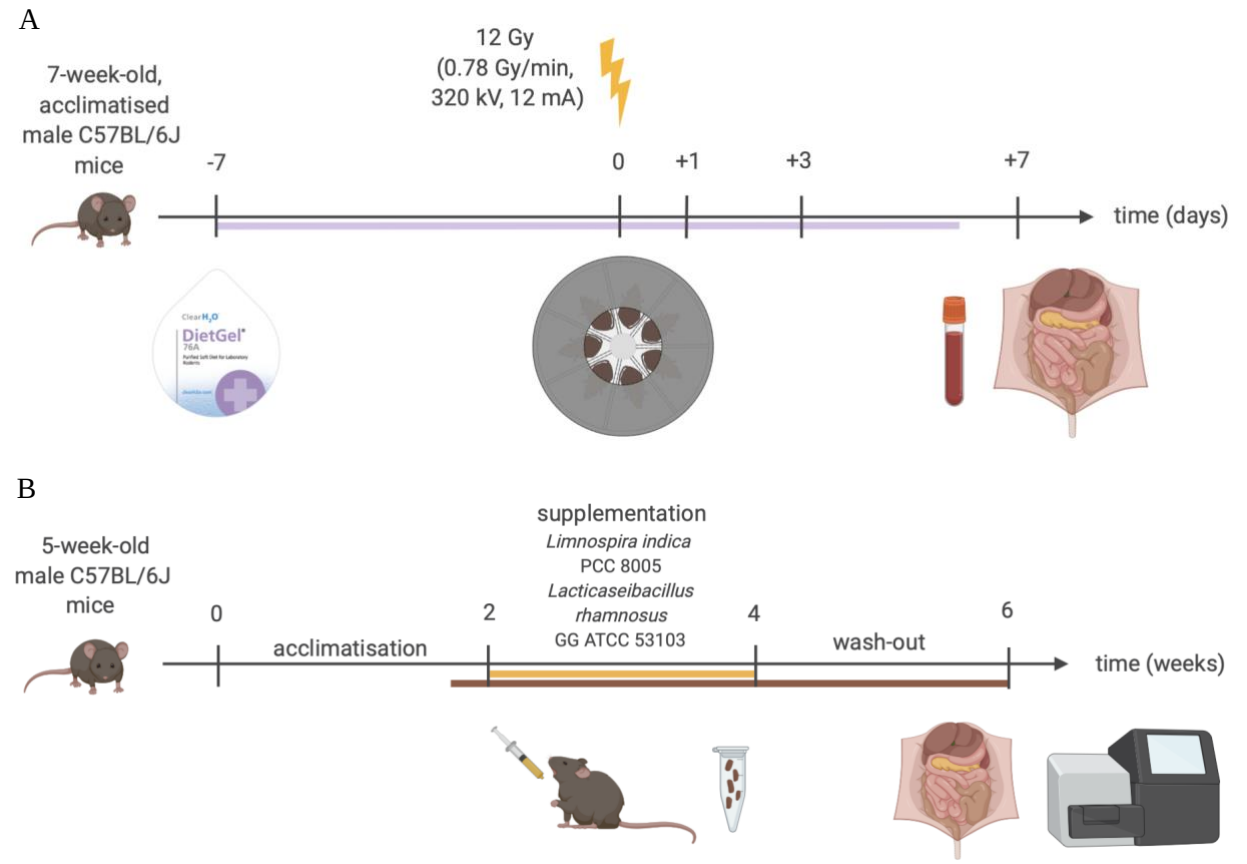

Fig. S1 – Experimental design of the present study. (A) Irradiation of healthy, non-supplemented mice. Mice were given diet gels from seven days before (sham-)irradiation until six days after (purple line). Food intake was measured six days before/after (sham-)irradiation. On the seventh day, mice were starved for the FITC-dextran assay. (B) Supplementation of healthy, non-irradiated mice. Mice were supplemented over the course of two weeks (yellow line). Faeces were collected from two days before the start of supplementation until the end of the wash-out period (brown line).

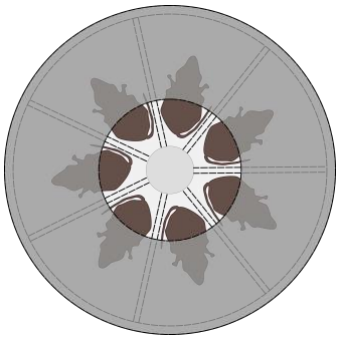

Fig. S2 – Mouse pie cage setup. This figure represents the "pie structure" setup used to perform local pelvic irradiation (view from above). Except for a 9 cm diameter hole in the center, the plexiglas structure (20 cm diameter, 5 cm hight) is entirely shielded with a 5 mm lead plate to perform local pelvic irradiation. Mice are placed in the pie cage in prone position.

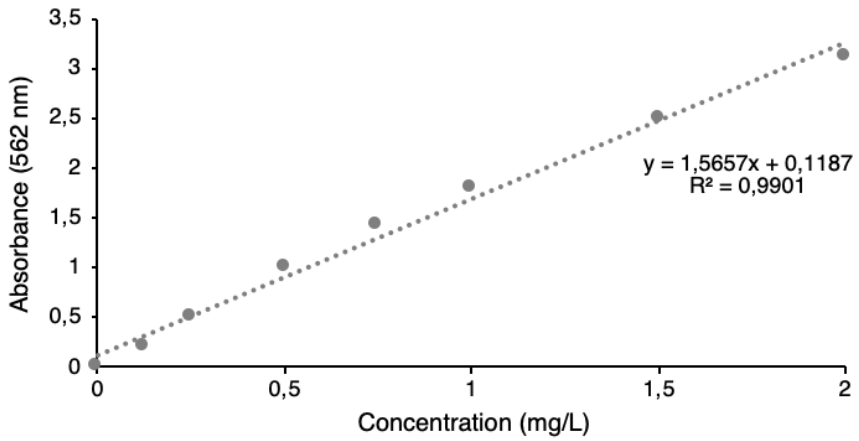

Fig. S3 – Standard curve of concentration versus absorbance using a BCA kit. The concentration of protein (mg/L) was determined using the equation y=1.5657 x + 0.1187 with an R^2 value of 0.9901, where y is the absorbance at 562 nm and x is the protein concentration.

Supplementary methods

***Limnospira* pigment and carotenoid extraction**

Twelve aliquots of cultures were collected by centrifugation (10,000 x g, 10 min, 4°C) (Centrifuge 5430 R, Eppendorf, Aarschot, Belgium). Pellets were stored at -80°C until further analysis. During pigment analyses, samples were kept in the dark. First, the pellets were resuspended in 1 mL of 0.05 M Na_2HPO_4 at pH 7. After five cycles of freezing in liquid nitrogen and thawing at 37°C, samples were incubated with 100 µL of lysozyme (100 mg mL^{-1}) for 30 min at 37°C (Thermomixer comfort, Eppendorf, Aarschot, Belgium). Next, the suspension was centrifuged at 13,000 x g for 10 min at 4°C (Centrifuge 5418 R, Eppendorf, Aarschot, Belgium). The absorbance of the supernatant was measured at wavelengths 615 and 652 nm using the Thermo Spectronic Unicam Aquamate Helios Spectrophotometer (Thermofisher Scientific, Merelbeke, Belgium). The concentration of phycocyanin (PC) and allophycocyanin (APC) were calculated as followed (150):

$$PC\ (mg\ mL^{-1}) = [A_{615} - 0.474 \times A_{652}]/5.34 \quad (1)$$

$$APC\ (mg\ mL^{-1}) = [A_{652} - 0.208 \times A_{615}]/5.09 \quad (2)$$

where A_{615} and A_{652} are the absorbance values measured at 615 and 652 nm, respectively.

The remaining pellet was washed three times using 0.05 M Na_2HPO_4 at pH 7. Total chlorophyll was extracted with 100% methanol as organic solvent. Additionally, sonication (3 x 10 s, amplitude 30%, 1 pulse rate) (UP50H Compact Ultrasonic Lab Homogenizer, Hielscher, Teltow, Germany) was performed to enhance chlorophyll extraction. The suspension was centrifuged at 13,000 x g and 4°C for 10 min (Centrifuge 5418 R, Eppendorf, Aarschot, Belgium) and the absorbance of supernatant was measured at 665 nm (Thermo Spectronic Unicam Aquamate Helios Spectrophotometer, Thermofisher Scientific, Merelbeke, Belgium). Chlorophyll (CHL) concentration was determined as followed (151):

$$CHL\ (mg\ mL^{-1}) = A_{665}/74.5 \quad (3)$$

where A_{665} is the absorbance value measured at 665 nm.

Carotenoid content was spectrophotometrically measured as previously described (152). Briefly, 6 culture samples were centrifuged at 4,000 x g for 10 min. The supernatant was decanted and an equal volume of methanol was added to the pellet. The suspension was incubated in a water bath at 55°C for 15 min (Thermomixer comfort, Eppendorf, Aarschot, Belgium), and subsequently centrifuged at 4,000 x g for 10 min (4°C). Absorbance was measured at 650, 665 and 452 nm using a using a CLARIOstar microplate reader (BMG Labtech, Isogen LifeScience, Utrecht, the Netherlands).
Concentration of carotenoids was calculated as followed:

$$Carotenoids\ (mg\ mL^{-1}) = 4.2\ A_{452} - [0.0246 \times (10.3\ A_{665} - 0.918\ A_{650})] \quad (4)$$

where A_{452}, A_{665} and A_{650} are the absorbance values measured at 452, 665 and 650 nm, respectively.

Protein extraction

The protein extraction protocol was adapted from Badri et al. (151). Twelve aliquots of cultures were obtained as mentioned above. Pellets were resuspended in 100 µL of 6 M guanidine chloride solution at pH 8.5 and kept on ice during the entire procedure. Cells were lysed by sonication (3 cycles of 10 s, 1 pulse rate). Next, samples were centrifuged at 16,000 x g for 15 min at 4°C. Total protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, Overijse, Belgium) in a 96 well plate. Briefly, 200 µL of BCA Working Reagent was added to 25 µL protein sample. Absorbance was measured at 562 nm using a CLARIOstar microplate reader (BMG Labtech, Isogen LifeScience, Utrecht, the Netherlands). Protein concentration was determined by comparison of the absorbance of unknown samples to the standard curve prepared using Bovine Serum Albumin (BSA) standards (Fig. S3).