Research Article

Effects of SBL-1 on Jejunal Microbiota in Total Body ^{60}Cobalt Gamma-Irradiated Mice - A Metagenomic Study with Implications towards Radioprotective Drug Development

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ABSTRACT

In earlier study, single dose of a standardized leaf extract of *Hippophae rhamnoides* (code SBL-1), @ 30 mg/kg body weight (b.w.) 30 min before whole body ^{60}Co-gamma-irradiation (10 Gy), rendered >90% survivors in comparison to zero survivor in non-SBL-1 treated ^{60}Co-gamma-irradiated (10 Gy) mice population. Present study investigated the modifying effects of SBL-1 on jejunal microbiota. 9 weeks old Swiss albino Strain'A' male mice were segregated as Untreated Control; ^{60}Co-gamma-irradiated (10 Gy); Drug alone (30 mg/kg b.w. SBL-1) and Drug + Radiation (30 mg/kg b.w. SBL-1, 30 min before 10 Gy). 16S rRNA gene amplicons, prepared using universal primers were sequenced on ABI3130, Genetic Analyzer. After removing vector contamination and chimeras, the sequences [GenBank Acc.No. KF681283 to KF681351] were taxonomically classified by using Sequence Match program, Ribosomal Database Project and by nucleotide-BLAST [E-value: 10, database: 16S rRNA gene sequences, Bacteria and Archea]. Phylogenetic Tree was prepared by MEGA, using maximum likelihood algorithm after sequence alignment by MUSCLE. Branch stability was assessed by bootstrap analysis. Rooted tree had *Thermus aquaticus* as out-group. Untreated Control and Drug alone groups had 100% *Lactobacillus*; Drug + Radiation had 89% *Lactobacillus* and 11% *Clostridium*; ^{60}Co-gamma-irradiated group had 55% *Cohaesibacter* (Alphaproteobacteria); 27% *Mycoplasma* (Tenericutes) and only 18% *Lactobacillus.* The radioprotective SBL-1 countered the radiation caused microbiota dysbiosis.

Keywords: 16S rRNA, *Lactobacillus*, Low LET radiation, Phylogenetic Tree, Seabuckthorn.

INTRODUCTION

The low linear energy transfer (LET) ionizing radiation are being increasingly used in diverse fields such as industry, medicine, warfare; and therefore, the threat of unwanted exposure to these radiation is ever increasing. The deep penetration property of low LET ionizing radiation damages multiple tissues simultaneously. Total body exposure to lethal dose (~ 10 Gy) of ^{60}Co-gamma-rays causes symptoms similar to gastro-intestinal (GI) tract syndrome and ultimately leads to death in 10-15 days. Presence of radiosensitive stem cells as well as proliferating epithelial cells in Crypts of Liberkuhn makes jejunum highly radiosensitive. The abdominal radiotherapy patients display diarrhea and loss of fluids as common ill-effects.[1] Studies, since long, have reported changes in *Lactobacillus* populations in GI tract of patients undergoing abdominal radiotherapy; but external supplementation with probiotic containing Lactobacilli was of little help. Although, no suitable explanation was put forth for lack of support by *Lactobacillus* supplements, presence of appropriate number of beneficial microbes in the intestine of patients undergoing radiotherapy, was considered important to improve the absorption and maintain electrolytic balance.[1-3]

Radiation induced oxidative stress caused by high flux of free radicals and consequent damage to DNA, lipid peroxidation of membranes, impaired antioxidant defense, are some of the factors, which cause disruption of intestinal mucosal barrier integrity and also inflammatory responses.[4] Therefore, most of the studies targeted to develop radiation countermeasures are focused on decreasing the radiation induced ROS for prevention of radiation injuries. However, despite rigorous studies for past many decades, world over, no antioxidant alone has demonstrated complete success in countering the ill effects of lethal doses of radiation. Moreover, till date, no medical radiation countermeasure has been approved as a safe drug for protection against lethal doses of ionizing radiation (~10 Gy or higher doses).[4] The severe toxicity displayed by WR2721, the only approved synthetic radio protective drug for radiotherapy patients, significantly limits its use.[5]

The enormity of challenges in developing radiation countermeasure necessitates the need for developing newer approaches and/or supplementing the existing approaches with more tests in the light of recent advances in knowledge, so that safe and effective radiation countermeasures can be developed. More recent studies on gut microbiota have demonstrated that gastrointestinal microbial communities not only help in the absorption of fluids and maintaining electrolytic balance, but also have roles in modifying the drug pharmacokinetics, pharmacodynamics and other body functions such as immune-system, anaerobic metabolism of peptides and proteins, protection against pathogens, recovery of metabolic energy, etc.[6-11] It is also understood that disturbances in the gut microbial population dynamics may influence the onset and progression of multiple diseases.[12, 13]

Previous studies demonstrated that administration of a single dose of standardized extract (code name SBL-1, prepared from leaves of *Hippophae rhamnoides* L., common name Seabuckthorn, family Elaeagnaceae), intraperitoneally @ 30 mg/kg body weight, 30 min before ^{60}Co-gamma-irradiation (10 Gy), rendered 94% survivors in experimental mice population for 30 days and beyond; while all non-SBL-1 treated-irradiated (10 Gy) animals died within 12 days after irradiation[14], suggesting the promising potential of SBL-1 as a radiation countermeasure.

Seabuckthorn is a deciduous and dioecious shrub with silvery leaves. It grows naturally as well as is cultivated in High altitude regions of north-west Himalayas (8000-11,000 feet) and can tolerate temperature variations from −25°C to +40°C. The rationale for choosing Seabuckthorn growing at high altitude regions was that natural exposure to high level of radiations, extreme temperature and humidity for thousands of years, may have enriched this plant with a combination of those secondary metabolites, which rendered protection from extreme environments. It was reasoned that such natural combination of bioactive compounds, acting synergistically, could be harnessed for developing medical radiation countermeasures for human use.[15] Seabuckthorn has a huge battery of bioactive compounds and anti-oxidants such as carotenoids, alpha-tocopherols, c-tocopherol, beta-tocotrienol, steroids, flavonoids, high amount of unsaturated fatty acids, vitamins A,C,E,K, minerals, tannins, polyphenols. The leaves of Seabuckthorn are extremely popular for their neutraceutical values and are consumed as constituents of health promoting drinks world over. In traditional Indian and Chinese medicinal systems the Seabuckthorn was recommended for treatment of gastric ailments, circulatory disorders, hepatic injuries and neoplasia.[16, 17] Earlier studies reported that on day 5, after total body ^{60}Co-gamma-irradiation (10 Gy), the epithelial layer of jejunal villi was highly discontinuous and the crypts were almost sterile;[18] the animals displayed malabsorption, diarrhea, inflammation, weight loss and died within 10-12 days.[14] On the other hand, in animals treated with radio protective dose of SBL-1 before ^{60}Co-gamma-irradiation (10 Gy), no discontinuity was observed in the mucosal layer and villi epithelium from day 5 onwards, crypts cellularity was similar to untreated controls; the animals were alive till day 30 and beyond.[18] The jejunal and crypts epithelial layer, since, is the niche of beneficial microbes, this study was undertaken to investigate effects of SBL-1 on microbiota in the jejunal mucosal epithelial layer. The 16S rRNA metagenomic approach was used to investigate the changes in both culturable and non-culturable microbiota dynamics in the experimental mice.

MATERIALS AND METHODS

Chemicals and reagents

The molecular biology grade chemicals were purchased from Merck, India; the DNase and RNase free plastic-ware were from Greiner, Germany. The aluminum chloride, Proteinase K, RNase and lysozyme were purchased from Sigma-Aldrich, USA. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Fermentas Germany**.** High performance thin layer chromatography (HPTLC) plates; RP-18 F254S (20x20 cm) were purchased from Merck, India. Reference standards Gallic acid ethyl ester, purity 98% w/w; and Rutin, purity 97%; were purchased from Acros Organics, Fischer scientific, USA; and Quercetin, purity 98%; was purchased from Fluka Biochemika, USA.

Plant extract (SBL-1) and its characterization

The plant extract was prepared and characterized as per procedure described earlier.[14] Briefly, the fresh green leaves of *Hippophae rhamnoides* L. (F. Elaeagnaceae), identified by ethno-botanists and confirmed as *Hippophae rhamnoides* [specimen records preserved at herbarium, Defence Institute for High Altitude Research (DIHAR), Leh, India, voucher specimen No SBTL-2006], were collected from Himalayas. The leaves were shade dried, powdered, soaked in distilled water. The supernatant was collected and lyophilized. The dried powder (yield 0.125 g/g) was coded as SBL-1. To ensure working with standardized extract and also to maintain the quality control, the HPTLC profile of SBL-1 was developed with reference to Quercetin dihydrate, Gallic Acid and Rutin. The separation of each compound was carried out on silica gel 60 F_{254} pre-coated TLC aluminum plates, while allowing linear ascending (9 cm) development at room temperature, in twin trough glass chamber saturated with suitably designed mobile phase. The comparisons with standards were made. Detailed procedure was as described earlier.[19]

Animals and experimental procedures

The 8-9 weeks old male, inbred, Swiss albino Strain 'A' mice, weighing 28±2 g, were used after the approval of Animal Experimentation Ethics Committee of the Institute. The animals were maintained under controlled environment at 26±2 °C; 12 h light/dark cycle and offered standard animal food (Golden feed, Delhi) as well as tap water *ad libitum.* All the animal experiments were conducted according to the guidelines of Committee for Protection and Care of Small Experimental Animals (CPCSEA) and as per the approved protocol. The animals were divided into four groups. Group I was Untreated Control and was administered vehicle (sterile water) only, group II was total body ^{60}Co-gamma-irradiated (10 Gy), group III was Drug alone (administered 30 mg/kg b.w. SBL-1 only) and group IV was Drug+Radiation (administered 30 mg/kg b.w. SBL-1 and after 30 min, animals were total body ^{60}Co-gamma-irradiated, 10 Gy). Each group had three mice and the experiment was repeated two times. The drug was dissolved in sterile water, filtered and administered intraperitoneally. For total (whole) body irradiation, each mouse was placed in a separate wire mesh container and was given one time

exposure to deliver 10 Gy radiation dose using ^{60}Co-gamma-ray source (GC-220, Atomic Energy of Canada Ltd., Canada, dose rate of 0.31 rad/sec). Fresh air was continuously circulated to avoid hypoxia.

On day 5 after irradiation, the mice were anaesthetized and sacrificed by cervical dislocation. The jejunum portion of the intestine was taken out after leaving approx. 4 cm segment from the pyloric sphincter side of stomach and placed in a cool sterile Petri-dish kept on ice. The contents were flushed out with the help of syringe loaded with 2 ml of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8) and collected in a tube. The jejunum was then cut opened longitudinally and epithelial mucosal layer was superficially scraped using round edged glass rod. Scraped material was pooled with previous collection from jejunum and contents were centrifuged to collect the pellet. The DNA was isolated using DNA isolation buffer (100 mM Tris/HCl pH 8.0, 50 mM EDTA pH 8.2 and 1.5 M NaCl) and lysozyme (final concentration of 5 mg/ml), as per the procedure described.[20] The extracted DNA was purified using QIAEX II Gel Extraction Kit (Qiagen GmbH) following manufacturer's instructions. Purity and the concentration of the extracted DNA was determined using spectrophotometer absorbance at 260/280 nm. DNA of samples showing absorbance ($A_{260/280}$ nm) ratios of 1.6-1.7 were accepted as pure. The integrity of DNA was determined after electrophoresis in a 0.8% agarose gel.

PCR amplification and amplicon purification

For the amplification of the 16S rRNA genes, two universal primers, 27f (5' AGAGTTTGATCATGGCTCAG 3', *E. coli* positions 8–27) and 1492r (5' TACGGTTACCTTGTTACGACTT 3', *E. coli* positions 1492–1513)[21], synthesized from Sigma-Aldrich, India, were used. The PCR reaction mixture contained 0.6 µM of each primer, template DNA, 10x PCR reaction buffer, 1.5 mM of $MgCl_2$ (Fermentas, Germany), 0.1 µg/µL of BSA (Sigma-Aldrich, USA), 0.2 mM of dNTPs (Promega Corporation, USA) and 1 Unit of Taq DNA polymerase (Fermentas, Germany) in a final volume of 25 µL. PCR amplification was performed using I-Cycler Thermal Cycler (BioRad Laboratories, USA). The program followed was, 95°C for 10 min, followed by 25 cycles consisting of 94°C for 30s, 50 °C for 1.5 min and 72°C for 1 min, and a final extension period of 72°C for 10 min.

To purify the PCR product, the amplified DNA was run on 1.2% agarose gel along with DNA molecular weight marker (O'GeneRuler Express DNA Ladder, Fermentas, Germany). The band of interest was cut and extracted using QIAEX II Gel Extraction kit (Qiagen, Germany). Competent cells were prepared as per procedure described.[22] The transformation efficiency of competent cells was checked on Luria agar plates containing Ampicillin, X-gal and IPTG. Competent cells with transformation efficiency of 10^6 transformants per µg DNA or more were utilized for further experiments. The purified amplicons were ligated and cloned into the pGEM-T Easy Vector System (Promega Corporation, USA) as per manufacturer's instructions. The white colonies growing on Luria agar plate (containing Ampicillin, X-gal and IPTG) were selected for further confirmation.

The plasmids were isolated from transformed cells as per the procedure described.[23] To check the presence of inserts, isolated recombinant plasmids were digested with EcoR1 (Promega Corporation, USA) in the presence of restriction buffer for 1 h at 37°C as per the information supplied with the restriction enzyme. The inserts were then separated by electrophoresis on 1.2% agarose gel containing molecular weight marker.

Sequencing of Inserts and Bioinformatics Analysis

Sequencing and subsequent data analysis was performed on ABI 3130, Genetic Analyzer using software 'Sequence Analysis_v5.2'. All near-full-length sequences were edited to exclude the PCR primer-binding sites using online tool 'VecScreen v7.1' (https://www.ncbi.nlm.nih.gov/tools/vecscreen/). After removing vector contamination, sequences were tested for possible chimeric artifacts using the DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu/ FindChimerasOutputs.html).[24] The non-chimeric sequences were analyzed for taxonomical classification using Sequence Match program of the Ribosomal Database Project (RDP), release 11, (http://rdp.cme.msu.edu/index.jsp, 2,809,406 16S rRNA gene sequences). The taxonomic classifications assigned by RDP for sequences having 's_ab' score ≥ 0.85 were adopted. Since RDP can have error up to 20% for certain regions and read lengths another tool, the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used, which is reported to achieve accuracy of 100%.[25] All the sequences were, therefore, also classified using RDP as well as nucleotide BLAST [E-value: 10, database: 16S rRNA gene sequences (Bacteria and Archea)].

All those sequences, which failed to achieve s_ab score of ≥ 0.85 after sequence match with RDP, but showed more than 85% 'maximum identity' and 'query coverage' after BLAST, were considered for further phylogenetic studies. Sequences which neither had s_ab score of ≥ 0.85 after RDP sequence match, nor had 85% 'maximum identity' as well as 'query coverage' after BLAST, were not included in the phylogenetic studies.

For phylogeny studies the sequences were aligned using the multiple sequence alignment program "MUltiple Sequence Comparison by Log- Expectation" (MUSCLE).[26] The MUSCLE alignment file (in fasta format) was used as input for the MEGA (v5.2) software to determine the phylogeny.[27] Phylogenetic trees were generated based on the maximum likelihood algorithm using the MEGA package. Branch stability was assessed by bootstrap analysis (1000 replicates) using the algorithms available in the MEGA package. *Thermus aquaticus* (GenBank Accession: EU682501.1) was used as the outgroup to construct rooted tree. The sequences were

submitted to GenBank repository (https://www.ncbi .njm.nih.gov/genbank/).

RESULTS

Characterization of SBL-1

The concentration of three bioactive and marker constituents of SBL-1 (Gallic acid ethyl ester, Quercetin dihydrate and Rutin trihydrate), as quantified by HPTLC, were found to be 12.09, 4.7 and 8.7 mg/g respectively. The HPTLC chromatogram of the Gallic acid ethyl ester, Quercetin dihydrate and Rutin trihydrate, as separated from SBL-1, is presented in Figure 1. For reference pure compounds were used. Gallic acid ethyl ester and Quercetin dihydrate are known for their anti-oxidant properties. Rutin is reported to have tissue regenerative properties.

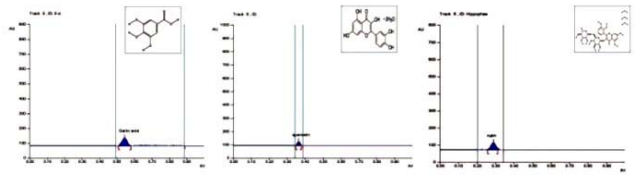

Figure 1: (a) HPTLC chromatogram of gallic acid ethyl ester, (b) chromatogram of quercetin dihydrate, (c) chromatogram of rutin trihydrate from SBL-1.

Isolation of recombinant plasmids and *in silico* analysis

The ethidium bromide stained agarose gel electrophoresis pictures of some of the representative recombinant plasmids, after digestion with EcoR1, are presented in Figure 2.

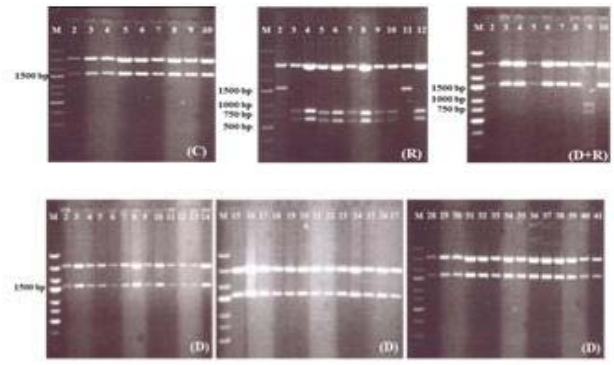

Figure 2: Agarose gel pictures of recombinant clones in pGEM-T Easy vector as seen after restriction digestion with EcoR1 (stained with ethidium bromide and visualized at 254 nm. The total DNA was isolated from jejunum of mice belonging to different treatment groups viz. untreated control (C); ^{60}Co-gamma-irradiated (R); Drug alone (D); Drug + Radiation (D+R). The clones were prepared from amplified DNA sequence of 16S rRNA gene. Lane 1 in all gels contained the marker (M), molecular weight is mentioned in base pairs. The clone numbers, as assigned by GenBank, were KF681283 to KF681291 in Lane 2-10 (C); KF681332 to F681342 in Lane 2-12 (R); KF681343 to KF681351 in Lane 2-10 (D+R); KF681292 to KF681331 in Lane 2-41 (D).

The sequences, which were free from vector contamination, chimeric sequences as well as were of high quality (69 in number), were submitted to GenBank (https://www.ncbi.njm.nih.gov/genbank/) and the GenBank accession numbers were from KF681283 to KF681351. The classification up to genus level, of all 69 sequences as determined by using nucleotide BLAST alignment tool [E-value (expectation value): 10, database: 16S ribosomal RNA sequences (Bacteria and Archea)] of NCBI as well as determined by using RDP database release 11, is presented in Table 1. The RDP classification up to genus level complemented the Nucleotide BLAST classification in all treatment groups except for the group containing sequences from ^{60}Co-gamma-irradiated animals. Genus *Lactobacillus* (Firmicutes) dominated in 3 treatment groups (Untreated Control, Drug alone Drug + Radiation). In Untreated Control and Drug alone group genus *Lactobacillus* had 100% share of the total population. In Drug + Radiation group the genus *Lactobacillus* had the 89% share of the total population while *Clostridium* genus had a share of 11%. In ^{60}Co-gamma-irradiated group, the genus *Cohaesibacter and* endosymbiont of Acanthamoeba (Alphaproteobacteria) dominated and had 55% share of the total population; *Mycoplasma* (Tenericutes) had 27% share and *Lactobacillus* had 18% share (Figure 3).

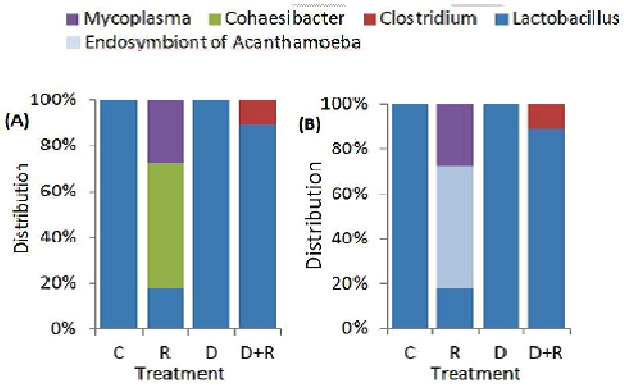

Figure 3: Bar graph shows changes in population of Firmicutes (*Lactobacillus* and *Clostridium*), Alphaproteobacteria (Endosymbiont of Acanthamoeba and *Cohaesibacter*) and Tenericutes (*Mycoplasma*) in jejunum of mice belonging to different treatment groups viz. untreated control (C); ^{60}Co-gamma-irradiated (R); Drug alone (D); Drug + Radiation (D+R).

(A) Shows distribution, obtained after using NCBI database and **(B)** shows distribution, obtained after using RDP database.

All 69 sequences were used for subsequent phylogenetic analysis. It was observed that 11 recombinant clone sequences [KF681286 (C); KF681322 and KF681330 (D); KF681336 to KF681340 and KF681342 (R); KF681346 and KF681350 (D+R)] showed ≤98% sequence similarity to existing 16S rRNA gene sequences in the GenBank and RDP databases. These clones could be the uncharacterized bacterial species (Table 2).

The phylogenetic tree prepared after analysis of DNA sequence of 16S rRNA isolated from the jejunum of mice belonging to different treatment groups is presented in Figure 4. Evolutionary history was inferred by using Maximum Likelihood method based on the Kimura 2-parameter model of MEGA package.[27]

Table 1: Taxonomical classification of recombinant clones based on sequence similarities, using SeqMatch tool of RDP database and BLAST tool of NCBI (16S rRNA Bacteria and Archaea) database. The clones were prepared from amplified 16S ribosomal DNA of jejunum of mice belonging to different treatment groups viz. untreated control (C); ^{60}Co-gamma-irradiated (R); SBL-1 (drug) treated (D); SBL-1 treated and irradiated (D+R).

Classification				RDP				NCBI			
				RDP SeqMatch (Isolates)				BLAST (16S rRNA Bacteria and Archaea)			
				C	R	D	D+R	C	R	D	D+R
Lineage	Genus	Species	Strain								
Domain: Bacteria, Phylum: Firmicutes, Class: Bacilli, Order: Lactobacillales, Family: Lactobacillaceae	*Lactobacillus*	*taiwanensis*	BCRC17755	8	-	34	-	9	-	39	-
		johnsonii	Is87	1	-	2	-	-	-	-	-
			NCC 533	-	-	-	-	-	2	1	1
			DPC 6026	-	1	-	-	-	-	-	-
			Lj16	-	1	1	1	-	-	-	-
		hominis	CRBIP 24.179	-	-	1	-	-	-	-	-
		crispatus	ST1	-	-	-	-	-	-	-	5
		gallinarum	ATCC 33199	-	-	-	1	-	-	-	1
		helveticus	DPC 4571	-	-	-	-	-	-	-	1
			KLDS 1.0601	-	-	-	1	-	-	-	-
		sp. B164	-nd-	-	-	-	5	-	-	-	-
		sp. AD102	-nd-	-	-	2	-	-	-	-	-
Total Genus *Lactobacillus*				9	2	40	8	9	2	40	8
Domain: Bacteria, Phylum: Firmicutes, Class: Clostridia, Order: Clostridiales, Family: Clostridiaceae	*Clostridium*	*perfringens*	13	-	-	-	-	-	-	-	1
		sp.ND2	-nd-	-	-	-	1	-	-	-	-
Total Genus *Clostridium*				-	-	-	1	-	-	-	1
Domain: Bacteria, Phylum: Proteobacteria, Class: Alphaproteobacteria, Order: Rhizobiales, Family: Cohaesibacteraceae	*Cohaesibacter*	*gelantinilyticus*	CL-GR15	-	-	-	-	-	6	-	-
Total Genus *Cohaesibacter*				-	-	-	-	-	6	-	-
Domain: Bacteria, Phylum: Proteobacteria, Class: Alphaproteobacteria, Unclassified Alphaproteobacteria	Endosymbiont of Acanthamoeba	*sp.UWET39*	-nd-	-	3	-	-	-	-	-	-
		sp.KA/E9	-nd-	-	3	-	-	-	-	-	-
Total Endosymbiont of Acanthamoeba				-	6	-	-	-	-	-	-
Domain: Bacteria, Phylum: Tenericutes, Class: Mollicutes, Order: Mycoplasmatales, Family: Mycoplasmataceae	*Mycoplasma*	*sualvi*	Mayfield B	-	3	-	-	-	3	-	-
Total (belongs to Genus *Mycoplasma*)				-	3	-	-	-	3	-	-

Table 2: Individual clone s_ab (seqmatch score) and maximum identity as derived from RDP database and NCBI database respectively

Treatment	Accession No	RDP SeqMatch	S_ab score	BLAST (16S rRNA)	Max Score	Query Coverage	Max identity
Untreated control (C)	KF681283	*Lactobacillus taiwanensis*; BCRC 17755	0.967	*Lactobacillus taiwanensis* BCRC 17755	2536	100%	99%
	KF681284	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2619	100%	99%
	KF681285	*Lactobacillus taiwanensis*; BCRC 17755	0.991	*Lactobacillus taiwanensis* BCRC 17755	2612	100%	99%
	KF681286	*Lactobacillus taiwanensis*; BCRC 17755	0.902	*Lactobacillus taiwanensis* BCRC 17755	2427	100%	98%
	KF681287	*Lactobacillus johnsonii*; Is87	0.980	*Lactobacillus taiwanensis* BCRC 17755	2488	99%	99%

	KF681288	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2636	100%	99%
	KF681289	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2634	100%	99%
	KF681290	*Lactobacillus taiwanensis*; BCRC 17755	0.987	*Lactobacillus taiwanensis* BCRC 17755	2641	100%	99%
	KF681291	*Lactobacillus taiwanensis*; BCRC 17755	0.975	*Lactobacillus taiwanensis* BCRC 17755	2604	100%	99%
SBL-1 (drug) treated (D)	KF681292	*Lactobacillus taiwanensis*; BCRC 17755	0.975	*Lactobacillus taiwanensis* BCRC 17755	2603	100%	99%
	KF681293	*Lactobacillus taiwanensis*; BCRC 17755	1.000	*Lactobacillus taiwanensis* BCRC 17755	2641	100%	100%
	KF681294	*Lactobacillus taiwanensis*; BCRC 17755	0.989	*Lactobacillus taiwanensis* BCRC 17755	2599	100%	99%
	KF681295	*Lactobacillus taiwanensis*; BCRC 17755	0.985	*Lactobacillus taiwanensis* BCRC 17755	2566	100%	99%
	KF681296	*Lactobacillus taiwanensis*; BCRC 17755	1.000	*Lactobacillus taiwanensis* BCRC 17755	2440	100%	100%
	KF681297	*Lactobacillus taiwanensis*; BCRC 17755	0.987	*Lactobacillus taiwanensis* BCRC 17755	2560	100%	99%
	KF681298	*Lactobacillus sp. AD102*	0.954	*Lactobacillus taiwanensis* BCRC 17755	2407	100%	99%
	KF681299	*Lactobacillus taiwanensis*; BCRC 17755	0.997	*Lactobacillus taiwanensis* BCRC 17755	2619	100%	99%
	KF681300	*Lactobacillus taiwanensis*; BCRC 17755	0.998	*Lactobacillus taiwanensis* BCRC 17755	2717	100%	99%
	KF681301	*Lactobacillus taiwanensis*; BCRC 17755	0.987	*Lactobacillus taiwanensis* BCRC 17755	2713	99%	99%
	KF681302	*Lactobacillus taiwanensis*; BCRC 17755	0.994	*Lactobacillus taiwanensis* BCRC 17755	2684	100%	99%
	KF681303	*Lactobacillus taiwanensis*; BCRC 17755	0.985	*Lactobacillus taiwanensis* BCRC 17755	2700	100%	99%
	KF681304	*Lactobacillus taiwanensis*; BCRC 17755	0.984	*Lactobacillus taiwanensis* BCRC 17755	2553	100%	99%
	KF681305	*Lactobacillus johnsonii*; Lj16	0.967	*Lactobacillus taiwanensis* BCRC 17755	2615	99%	99%
	KF681306	*Lactobacillus taiwanensis*; BCRC 17755	0.994	*Lactobacillus taiwanensis* BCRC 17755	2723	100%	99%
	KF681307	*Lactobacillus taiwanensis*; BCRC 17755	0.988	*Lactobacillus taiwanensis* BCRC 17755	2721	100%	99%
	KF681308	*Lactobacillus taiwanensis*; BCRC 17755	0.985	*Lactobacillus taiwanensis* BCRC 17755	2628	100%	99%
	KF681309	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2625	100%	99%
	KF681310	*Lactobacillus taiwanensis*; BCRC 17755	0.988	*Lactobacillus taiwanensis* BCRC 17755	2678	100%	99%
	KF681311	*Lactobacillus sp. AD102*	1.000	*Lactobacillus taiwanensis* BCRC 17755	2442	100%	100%
	KF681312	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2625	100%	99%
	KF681313	*Lactobacillus taiwanensis*; BCRC 17755	0.992	*Lactobacillus taiwanensis* BCRC 17755	2717	99%	99%
	KF681314	*Lactobacillus taiwanensis*; BCRC 17755	0.978	*Lactobacillus taiwanensis* BCRC 17755	2686	100%	99%

	KF681315	*Lactobacillus taiwanensis*; BCRC 17755	0.989	*Lactobacillus taiwanensis* BCRC 17755	2529	99%	99%
	KF681316	*Lactobacillus taiwanensis*; BCRC 17755	0.989	*Lactobacillus taiwanensis* BCRC 17755	2582	100%	99%
	KF681317	*Lactobacillus taiwanensis*; BCRC 17755	0.970	*Lactobacillus taiwanensis* BCRC 17755	2573	98%	99%
	KF681318	*Lactobacillus taiwanensis*; BCRC 17755	0.979	*Lactobacillus taiwanensis* BCRC 17755	2617	100%	99%
	KF681319	*Lactobacillus johnsonii*; ls87	0.995	*Lactobacillus taiwanensis* BCRC 17755	2514	100%	99%
	KF681320	*Lactobacillus taiwanensis*; BCRC 17755	0.990	*Lactobacillus taiwanensis* BCRC 17755	2634	100%	99%
	KF681321	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2649	100%	99%
	KF681322	*Lactobacillus taiwanensis*; BCRC 17755	0.864	*Lactobacillus taiwanensis* BCRC 17755	2385	99%	97%
	KF681323	*Lactobacillus taiwanensis*; BCRC 17755	0.932	*Lactobacillus taiwanensis* BCRC 17755	2484	100%	99%
	KF681324	*Lactobacillus johnsonii*; ls87	0.994	*Lactobacillus taiwanensis* BCRC 17755	2490	99%	99%
	KF681325	*Lactobacillus taiwanensis*; BCRC 17755	0.987	*Lactobacillus taiwanensis* BCRC 17755	2643	100%	99%
	KF681326	*Lactobacillus taiwanensis*; BCRC 17755	0.943	*Lactobacillus taiwanensis* BCRC 17755	2501	100%	99%
	KF681327	*Lactobacillus taiwanensis*; BCRC 17755	0.953	*Lactobacillus taiwanensis* BCRC 17755	2567	99%	99%
	KF681328	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2632	100%	99%
	KF681329	*Lactobacillus taiwanensis*; BCRC 17755	0.934	*Lactobacillus taiwanensis* BCRC 17755	2562	100%	99%
	KF681330	*Lactobacillus hominis*; CRBIP 24.179	0.943	*Lactobacillus johnsonii* NCC 533	2519	99%	98%
	KF681331	*Lactobacillus taiwanensis*; BCRC 17755	0.995	*Lactobacillus taiwanensis* BCRC 17755	2643	100%	99%
^{60}Co-gamma-irradiated (R)	KF681332	*Lactobacillus johnsonii*; Lj16	0.988	*Lactobacillus johnsonii* NCC 533	2595	100%	99%
	KF681333	*Mycoplasma sualvi*; Mayfield B	0.948	*Mycoplasma sualvi* Mayfield B	2420	100%	99%
	KF681334	*Mycoplasma sualvi*; Mayfield B	0.920	*Mycoplasma sualvi* Mayfield B	2523	99%	99%
	KF681335	*Mycoplasma sualvi*; Mayfield B	0.923	*Mycoplasma sualvi* Mayfield B	2542	99%	99%
	KF681336	endosymbiont of Acanthamoeba *sp. UWET39*	0.509	*Cohaesibacter gelatinilyticus* CL-GR15	1267	91%	86%
	KF681337	endosymbiont of Acanthamoeba *sp. UWET39*	0.514	*Cohaesibacter gelatinilyticus* CL-GR15	1290	91%	86%
	KF681338	endosymbiont of Acanthamoeba *sp. KA/E9*	0.510	*Cohaesibacter gelatinilyticus* CL-GR15	1303	91%	86%
	KF681339	endosymbiont of Acanthamoeba *sp. UWET39*	0.509	*Cohaesibacter gelatinilyticus* CL-GR15	1290	91%	86%

	KF681340	endosymbiont of Acanthamoeba *sp. KA/E9*	0.513	*Cohaesibacter gelatinilyticus* CL-GR15	1303	90%	86%
	KF681341	*Lactobacillus johnsonii* DPC 6026	0.960	*Lactobacillus johnsonii* NCC 533	2562	100%	99%
	KF681342	endosymbiont of Acanthamoeba *sp. KA/E9*	0.514	*Cohaesibacter gelatinilyticus* CL-GR15	1303	90%	86%
SBL-1 treated and irradiated (D+R)	KF681343	*Lactobacillus johnsonii* Lj16	0.981	*Lactobacillus johnsonii* NCC 533	2551	100%	99%
	KF681344	*Lactobacillus sp. B164*	0.970	*Lactobacillus crispatus* ST1	2555	100%	99%
	KF681345	*Lactobacillus gallinarum*; ATCC 33199	0.964	*Lactobacillus gallinarum* ATCC 33199	1358	100%	99%
	KF681346	*Lactobacillus helveticus*; KLDS 1.0601	0.742	*Lactobacillus helveticus* DPC 4571	623	96%	93%
	KF681347	*Lactobacillus sp. B164*	0.964	*Lactobacillus crispatus* ST1	2549	100%	99%
	KF681348	*Lactobacillus sp. B164*	0.940	*Lactobacillus crispatus* ST1	2483	100%	99%
	KF681349	*Lactobacillus sp. B164*	0.942	*Lactobacillus crispatus* ST1	2492	99%	99%
	KF681350	*Clostridium sp.ND2*	0.996	*Clostridium perfringens* 13	2143	100%	95%
	KF681351	*Lactobacillus sp. B164*	0.970	*Lactobacillus crispatus* ST1	2566	100%	99%

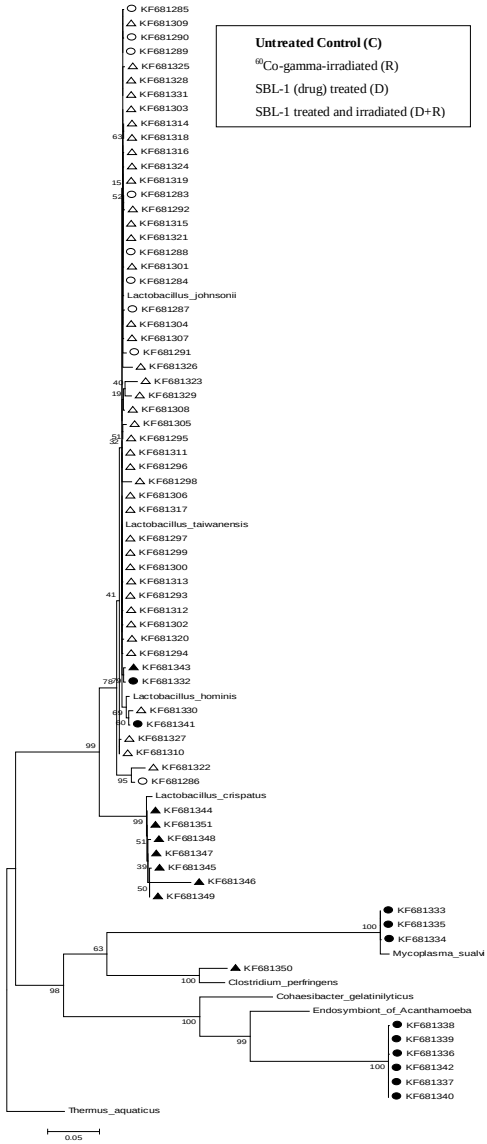

Figure 4: The phylogenetic tree prepared after analysis of DNA sequence of 16S rRNA isolated from the jejunum of mice belonging to different treatment groups viz. untreated control (C); ^{60}Co-gamma-irradiated (R); Drug alone (D); Drug + Rdiation (D+R). The phylogenic tree with highest log likelihood (-9041.7604) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. *Thermus aquaticus* (EU682501.1) was used as an out group. Bootstrap values shown at the branches are based on 1000 replicates. Values of 95% or higher were considered significant. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.2151)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The scale bar represents genetic distance (05 substitutions per 100 nucleotides). Reference sequences were from GenBank – *Lactobacillus johnsonii* (EU381129.1), *Lactobacillus taiwanensis* (NR_044507.1), *Lactobacillus hominis* (FR681902.1), *Lactobacillus crispatus* (NR_074986.1), *Mycoplasma sualvi* (NR_041846.1), *Clostridium perfringens* (NR_074482.1), *Cohaesibacter gelatinilyticus* (NR_043777.1), Endosymbiont of *Acanthamoeba* (AF132139.1).

The analysis involved 78 nucleotide sequences (69 recombinant clones+8 reference sequences+1 out group). Condon positions included were 1st+2nd+3rd+non-coding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. The jejunal bacterial population of untreated animals (C), SBL-1 treated animals (D) and SBL-1+irradiated (D+R) animals belonged to *Lactobacillus* genus, although there were differences in the species. Bacterial population of animals which were lethally irradiated showed significant divergence in comparison to animals of group C, D and D+R at genus level. Majority of

clones of irradiated animals apparently belonged to *Mycoplasma*, *Cohaesibacter* and endosymbionts of Acanthamoeba.

DISCUSSION

Increased usage of ionizing radiation in many walks of life has increased the risk of radiation injuries to human population. This has necessitated the need of developing an effective and safe radiation counter-measures for human use. Even after decades of research, this need has not been fulfilled. The synthetic compounds have failed miserably due to severe toxicity. The only drug WR2721, which was approved for human use as an adjuvant to radiotherapy, has serious toxic effects.[5] Herbal drugs are emerging as alternate choice because of their relatively non-toxic nature. However, the issues pertaining to standardization and quality control of herbal drugs pose multiple challenges to the scientific community. The precise quantification of three marker compounds Gallic acid ethyl ester, Quercetin dihydrate and Rutin trihydrate (Figure 1) in the promising radioprotective extract of Seabuckthorn (coded as SBL-1)[14], was to ensure the quality control of SBL-1, used in this study.

The failures of potential radiation countermeasures due to their associated toxicity and/or inadequate efficacy in humans are important reasons, which are compelling enough to consider deploying additional/newer approaches and/or increasing the ambit of pre-clinical tests before zeroing on to the medical preparations for clinical investigations. The gut microbiome project has shown beyond doubt that intestine is not merely an organ for absorption of nutrients and disposal of wastes, but actually performs many more functions owing to the presence of large number of highly specific microbes. The microbiota of intestine, besides participating in various physiological activities, such as appetite control, energy balance, allergies, behavioural perturbations, immune response;[28] plays an important role in determining the final outcome of a drug. Any treatment leading to dysbiosis of intestinal microbiota is therefore, expected to influence the distribution, metabolism, success, failure, efficacy as well as toxicity of the drug. Keeping in view the advancements of knowledge from the ongoing microbiome projects, we thought it was necessary to include the intestinal microbiota studies in our radioprotective drug development programme.

Ionizing radiations cause mucosal atrophy and disturb the mucosal microbiota leading to translocation of microorganisms or microbial products through the mucosa into blood circulation which could lead to sepsis and life threatening complications.[29] It was therefore, considered important to investigate the effects of radio protective drug SBL-1 on the microbiota of intestine when administered at radioprotective concentration before irradiation with lethal doses of ^{60}Co-gamma-radiation (10 Gy). The jejunum, since, is the most radiosensitive portion of the intestine; the pilot study was performed on jejunal microbiota. Because of acidic pH and relatively higher concentration of oxygen in jejunum, in comparison to distal portions of intestine, presence of facultative gram positive, non-spore forming, aerotolerant, acidophilic anaerobic bacteria belonging to Lactobacillaceae family is frequently reported in jejunum.[30] The abundance of Lactobacilli in unirradiated group of animals (Table 1, 2, Figure 4, 5), is in line with the previous studies. Total body exposure to ^{60}Co-gamma-ray (10 Gy) disturbed the microbiota distribution in jejunum thereby creating dysbiosis. Only 18% clones had shown match with *Lactobacillus*. The presence of endosymbionts of Acanthamoeba was predominant (55%). Further 27% match was with *Mycoplasma*. The dysbiosis in the jejunum of irradiated mice could be due to the displacement of microbes present in the distal portion of intestine. Presence of Alphaproteobacteria, Tenericutes and Clostridia were reported in large intestine as well as the distal ileum[30], Mollicutes were reported in the colon as well as faeces of rodents.[30,31] The radiation injuries such as leakage of vasculature, erosion of epithelial layer and change in pH, may be some of the important reasons facilitating the migration of Alphaproteobacteria, Clostridia and Mollicutes in the jejunum from the lower intestine. The classification based on 16S ribosomal RNA sequence as performed in this study (Figure 4) clearly demonstrated that clones detected in the irradiated animals were evolutionarily different than the clones which were detected in the untreated animals. Unlike Lactobacilli detected in unirradiated control, the microbes belonging to Alphaproteobacteria, Clostridia and Mollicutes were not the native inhabitant of jejunum. A recent study[32], reported increase in population of 12 members of *Bacteroidales*, *Lactobacillaceae*, and *Streptococcaceae* in rat faeces after radiation exposure at lethal doses. The same study also reported decrease in population of *Clostridiaceae* and *Peptostreptococcaceae* family members.

Treatment with SBL-1 alone apparently increased the population of Lactobacilli in jejunum. This observation draws strength from the increased number of transformation frequency detected in drug alone group (Figure 2). Further, NCBI classification and bootstrapping values in phylogenetic analysis also showed that the clones isolated from drug alone group were Lactobacilli and were not significantly different in comparison to untreated control; although some newer species of Lactobacilli were detected. The microbiota of intestine participates in the metabolism of polyphenols and flavonoids.[33,34] SBL-1 is rich in polyphenols, flavonoids, tannins and proanthocyanidins.[14] The increase in the population of Lactobacilli in animals treated with SBL-1 alone could be to facilitate the metabolism of various constituents of SBL-1 and maintain proper energy balance. The increased amount of fat in the diet of experimental animals was reported to bring out a change in the gut microbiota to ensure the energy balance.[10]

In comparison to radiation alone group, the animals treated with SBL-1 at radio protective concentration

before irradiation (10 Gy) preserved the Lactobacilli population to a large extent. As much as 89% clones had shown match with *Lactobacillus* genus. Complete absence of pathogenic bacteria belonging to *Mycoplasma* or endosymbiont of Acanthamoeba, as was observed in irradiated animals, indicated that pretreatment with SBL-1 protected the native Lactobacilli population of irradiated animals. Appearance of newer species of Lactobacilli such as *L. crispatus*, *L. gallinarum*, *L. helveticus* in drug + radiation group of animals needs further investigation. None-the-less, this study shows that in addition to the anti-oxidative, tissue regenerative, anti-inflammatory and other radiation protection mechanisms of SBL-1 reported in the previous studies,[14,35-37] this study showed that SBL-1 rendered radioprotection to the whole body irradiated animals by preserving the symbiosis state of native microbial population in jejunum. The native Lactobacilli population of the jejunum renders many beneficial effects to the host. It serves as a natural enhancer of cellular immune response.[38] *Lactobacillus salivarius* UCC118 was reported to protect the mice against infection with *Listeria monocytogenes.*[39] Lactibacilli was reported to stimulate NADPH oxidase 1 (Nox1)-dependent ROS generation and consequent cellular proliferation in intestinal stem cells.[40] To the best of our knowledge this study is the first one where a radio protective drug has shown to preserve the symbiosis of the native microbiota of the jejunum. The role of intestinal microbiota in multiple functions of body necessitates that drugs meant for complicated pathologies should be evaluated for their modifying effect on the intestinal microbiota.

CONCLUSION

This study showed that SBL-1 rendered radioprotection to the whole body irradiated animals by preserving the symbiosis state of native microbial population in jejunum.

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