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Rice Bran Extracts Inhibit Invasion and Intracellular Replication of *Salmonella typhimurium* in Mouse and Porcine Intestinal Epithelial Cells

Irfan A Ghazi[1,7#], Iman Zarei[1,9], Job O Mapesa[1,8#], Jessie R Wilburn[2], Jan E Leach[3], Sangeeta Rao[4], Corey D Broeckling[5], Anna McClung[6] and Elizabeth P Ryan[1*]

[1]*Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, USA*
[2]*Department of Food Science and Nutrition, Colorado State University, Fort Collins, CO 80523, USA*
[3]*Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523, USA*
[4]*Animal Population Health Institute, Department of Clinical Sciences, Colorado State University, Fort Collins, CO 80523, USA*
[5]*Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO 80523, USA*
[6]*USDA-Agricultural Research Service, Dale Bumpers National Rice Research Center, Stuttgart, AR 72160, USA*
[7]*Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana, India*
[8]*Department of Human Nutrition and Dietetics, Kenya Methodist University, Nairobi, Kenya*
[9]*Institute of Human Nutrition and Food, College of Human Ecology, University of the Philippines Los Baños, Los Baños 4031, Laguna, Philippines*
[#]*Contributed equally to this work*

Abstract

Dietary rice bran supplementation has been shown to inhibit *Salmonella* fecal shedding in animals. The aim of this study was to determine if bran extracts from two distinct rice varieties, Lijiangxintuanheigu (LTH) and Sanhuangzhan-2 (SHZ-2), differentially inhibit *Salmonella enterica* serovar Typhimurium invasion and intracellular replication. Rice bran extracts were tested *in vitro* using mouse small intestine epithelial (MSIE) and intestinal porcine epithelial cells (IPEC-J2). Fluorescent labeled *Salmonella* was detected using fluorescence microscopy and culture based methods. Non-targeted metabolomics using ultra-performance liquid chromatography- mass spectrometry (UPLC-MS) was performed on LTH and SHZ-2 rice bran extracts. LTH bran extract dose-dependently reduced entry and intracellular replication of *Salmonella* in both MSIE and IPEC-J2 cells when compared with SHZ-2. The rice bran metabolite profiling revealed significant variations between LTH and SHZ-2. LTH had higher total numbers of metabolites (429) versus SHZ-2 (407), with increased relative abundance of lipids (i.e., galactolipids and phospholipids) and flavonoids compared with SHZ-2. SHZ-2 had higher levels of dipeptides and phenylpropanoids. Distinct metabolite differences between LTH and SHZ-2 revealed rice bran components that may be responsible for blocking *Salmonella* invasion and intracellular replication. Metabolomics is a powerful phenotyping tool for identifying rice bran compounds with protective effects against pathogens. Future studies may identify the rice genes responsible for these bioactive rice bran metabolites distinguishing LTH from SHZ-2, and enable genetic selection for compounds as traits that offer important health and disease fighting benefits.

Keywords: Bacterial invasion and replication; Metabolomics; Rice bran; *Salmonella*; Small intestine

Abbreviations: CFU: Colony forming units; GFP: Green fluorescent protein; IPEC-J2: Intestinal porcine epithelial; LTH: Lijiangxintuanheigu; MSIE: Mouse small intestine epithelial; RB: Rice bran; RBE: Rice bran extract; SHZ-2: Sanhuangzhan-2; UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry.

Introduction

The Center for Disease Control and Prevention estimates that 21 million *Salmonella* infections cause illnesses that result in more than 200,000 deaths globally each year [1,2]. Some reports suggest that *Salmonella* stimulates transcriptional reprogramming in host cells, produces pro-inflammatory cytokines, and promotes cytoskeletal rearrangements that contribute to diarrhea [3,4]. There are a number of barriers to treatment, including antimicrobial resistance, and limited access to and high costs of antibiotics [5]. We and others have previously demonstrated that dietary rice bran promotes resistance to *Salmonella enterica* serovar Typhimurium colonization in mice [6,7]. Additionally, bran from different rice varieties showed differential protection against *Salmonella* [8]. Recent studies also demonstrate that dietary rice bran protects against rotavirus diarrhea and promotes Th1-type immune responses to human rotavirus vaccine in gnotobiotic pigs [9,10]. While the antimicrobial activity of rice bran extracts for protection against diarrheal disease is tested on a number of pathogens, the effects of these rice bran extracts have not been previously tested for mechanisms of enteric pathogen invasion and intracellular replication.

Rice bran also delivers a distinct biochemical profile, including lipids, compared to other cereal grains that modulate immunity [11-14] and merits investigation for protective mechanisms against enteric pathogens [7,9,15-17]. A number of rice bran components (e.g., lipids and polyphenols) have been broadly investigated for protection against chronic diseases such as cancer [18-20], type II diabetes [21,22], and hyperlipidemia [23].

A majority of rice cultivars are grouped into the subspecies japonica and indica, with distinct traits for plant architecture, agronomic and physiological features [24]. Recent evidence also demonstrated that japonica and indica have distinctive seed metabolomes [25]. Given that rice bran varieties differently protect against *Salmonella* colonization [8], we chose to compare bran from two genetically diverse varieties, Lijiangxintuanheigu (LTH) and Sanhuangzhan-2 (SHZ-2) based on

***Corresponding author:** Elizabeth P Ryan, Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, USA, Tel: +19704911536; E-mail: E.P.Ryan@colostate.edu

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single nucleotide polymorphisms (SNPs) previously reported from a classified set of 20 rice varieties [26,27]. We hypothesized differential inhibition of *Salmonella enterica* serovar Typhimurium invasion and intracellular replication in small intestinal cells by rice bran extracts. To study differences between the rice bran types, LTH and SHZ-2, for protection against *Salmonella*, we applied non-targeted metabolomics.

Materials and Methods

Rice milling and heat stabilization

Two rice varieties, Lijiangxintuanheigu (LTH) -*Oryza sativa* Japonica- and Sanhuangzhan-2 (SHZ-2)- *Oryza sativa* Indica- were obtained from the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) Dale Bumpers National Rice Research Center, Stuttgart, AR, USA. LTH is a short grain cultivar with red bran, whereas SHZ-2 is a long grain cultivar with light brown bran [26,27] (Figure S1). Seed increase of SHZ-2 was performed at Stuttgart, AR and harvested in September 2011 while that of LTH was performed in Lajas, Puerto Rico and harvested in April 2012.

The seeds were cleaned and stored at 4°C until bran preparation. Rough rice was dehulled using a Yamamoto testing husker (Model: FC2K l), and bran was removed and collected using a Yamamoto test whitening machine (Rice pal VP-31T). Testing sieve No. 20 was used to separate bran from broken rice and hulls into a clean container. Once separated, milled bran was heat stabilized at 110°C for 6 min to prevent rancidity during storage. RB was stored at -20°C until further processing for metabolite analysis.

Rice bran extract (RBE) preparation

Non-targeted metabolomics was used to analyze rice bran extracts at Metabolon Inc. (Durham, NC). Sample preparation used the automated MicroLab STAR® system from Hamilton Company. A recovery standard was added prior to extraction for quality control. Rice bran was mixed with ice-cold 80% methanol. Chemically diverse metabolites were concentrated in the supernatant after vigorous shaking (Glen Mills GenoGrinder 2000) followed by centrifugation. Rice bran extracts were applied to UPLC-MS/MS with positive and negative ion mode electrospray ionization and for intestinal cell culture-based *Salmonella* infections. For the cellular assays, lyopholized rice bran extract was diluted in 1 ml medium, vortexed to dissolve compounds, and filtered through a sterile 0.45 µm pore size Whatman syringe filter (Cat # 6789-1304; Whatman Inc.). Dose response experiments were completed and final working concentrations of 1 mg/ml (MSIE) and 5 mg/ml (IPEC-J2) rice bran extract were used after assessing cell mortality (data not shown and as described in Ref. [6]).

Cell culture conditions

Mouse small intestine epithelial cells (MSIE): MSIE cells were a gift from Dr. Robert Whitehead at Vanderbilt University and the Ludwig Institute for Cancer Research [28]. MSIE cells were maintained on RPMI 1640 medium (Fischer Scientific) supplemented with 10% FBS (Cat # F0500A-HI; Atlas Biologicals), 1 × antibiotic/antimycotic (Cat # 15240-062; Life Technologies), 10 µM thioglycerol (Cat # M6145; Sigma), 1 µg/ml hydrocortisone (Cat # H0135; Sigma), 2.0 mM/ml L-glutamine (Hyclone Laboratories), 1 µg/ml regular human insulin (Novo Nordik), and 10 units/ml murine IFN-γ (Peprotech). Cells were grown in 75 cm^2 flasks, split at 80% confluence twice weekly, and the culture medium replaced every two days. Cells were maintained at 33°C with 5% CO_2 and 95% relative humidity. Trypan blue staining was used to evaluate cell viability. Trypsinized (0.25% Trypsin-EDTA; Life Technologies) cells were seeded in 24 well plates at a density of 5 × 10^4 cells/ml. After 48 h incubation, the culture medium was removed and fresh medium containing rice bran extracts added for 2 h. The cell monolayer was washed twice with phosphate buffered saline (PBS, pH 7.4) before *Salmonella* infection.

Intestinal porcine epithelial cells- jejunum 2 (IPEC-J2): IPEC-J2 cells were a gift from Dr. Lijuan Yuan at Virginia Polytechnique Institute and State University. IPEC-J2 cells were maintained on DMEM/F12 medium (Life Technologies) supplemented with 5% FBS, 1 × antibiotic/antimycotic, 2.0 mM/ml L-Glutamine, 5 µg/ml Insulin-Transferrin-Selenium (Life Technologies), and 5 ng/ml EGF recombinant human protein solution (Life Technologies). Cells were maintained at 37°C+5% CO_2, 95% relative humidity. Trypsinized cells were seeded in 24 well plates at a density of 1 × 10^5 cells/ml, after 48 h the culture medium was removed and fresh medium containing rice bran extracts added for 2 h. The cell monolayer was washed twice with PBS before *Salmonella* infection.

***Salmonella enterica* infection:** Green Fluorescent Protein (GFP) labeled *Salmonella enterica* serovar Typhimurium (strain 14028s) was a gift from Dr. Andres Vazquez-Torres at University of Colorado Denver. GFP *Salmonella* was prepared as previously described [6]. MSIE cells were infected with 100 MOI (Multiplicity of Infection) *Salmonella* in PBS and centrifuged at 1,200 × g for 2 min to promote bacterial interaction with cells. After 1 h of incubation the medium was removed and the cell monolayer washed twice with PBS to remove extracellular bacteria. The cells were incubated with medium containing 100 µg/ml gentamicin (Cat # G1397; Sigma) for 1 h, washed twice in PBS before a further incubation in medium containing 50 µg/ml gentamicin for 1 h (invasion) or 24 h (replication) to prevent extracellular *Salmonella* growth. The cells were washed twice with PBS and incubated for 30 min in medium containing 1 × DAPI/Texas red dye (Cat # ENZ-53005-C100; ENZO Life Sciences). Cells were washed twice with 1 × PBS to remove the excess staining solution before observing under a fluorescence microscope.

GFP *Salmonella* fluorescence (Quantification): Fluorescence was determined using Cytation 3 cell imaging multi-mode reader (BioTek, Winooski, VT, USA) with the following imaging modes at 20 × objective: Brightfield and Fluorescence (Excitation/Emission): DAPI (377/447 nm), GFP (469/525 nm), and CY5 (625/685 nm). Percent *Salmonella* infection was quantified by expressing the ratio of GFP *Salmonella* signal to cell nuclei count as a percentage [(GFP/Nuclei) × 100]. After fluorescence reading, the cells were lysed for bacterial enumeration.

Colony forming units (CFU): *Salmonella* growth was measured as follows: The epithelial cells were washed twice before lysed in buffer [1% TritonX-100 (Sigma) and 0.1% Sodium Dodecyl Sulphate (Sigma) in PBS] for 5 min to free the intracellular *Salmonella*. The released *Salmonella* cells were mixed and pipetted at 1 ml equivalent of cell lysate, and serially diluted 1:10 in 1 × PBS. 100 ml of each dilution factor was plated on MacConkey agar (BD Biosciences) supplemented with 50 µg/ml kanamycin (Lot # 085309; Fisher Scientific). After incubation for 16 h at 37°C, total *Salmonella* CFU/ml were enumerated.

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)

A Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap

mass analyzer operated at 35,000 mass resolution was used for non-targeted metabolomics. Each sample contained eight or more injection standards at known concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using same columns (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 µm). Rice bran extracts diluted in acidic conditions, were further eluted using water and methanol containing 0.1% formic acid from a C18 column. The same elution procedure was performed for basic extracts, however, ammonium bicarbonate was used instead of formic acid. The third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with ammonium formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion, and the scan range was from 80-1000 m/z.

Compound identification analysis

Metabolon's hardware and software were used to process the LC-MS data. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. The mass spectra of the metabolites were compared to the NIST (National Institute of Standards and Technology) library. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. More than 3000 commercially available purified standard compounds were acquired and registered into Metabolon's Laboratory Information Management System (LIMS) for determination of analytical characteristics. A data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. The relative abundance of each metabolite was further normalized by the mediumn obtained from entire dataset.

Statistical analysis

The CFU data was analyzed using GraphPad Prism 5 (GraphPad Software; San Diego California) in one-way ANOVA and the differences in sample means analyzed by Dunnett's Multiple Comparison Test ($p<0.05$). CFU data were transformed to $\log_{10}$ scale and presented as mean ± SEM of triplicate samples representative of at least three independent experiments. A t-test/f-test was conducted on mediumn-scaled normalized relative abundance of entire data set (LTH vs. SHZ-2) to determine whether the entire metabolite means between these varieties differ.

Results

Dose and time dependent effects from LTH and SHZ-2 rice bran extracts on *Salmonella* intracellular replication

MSIE and IPEC-J2 were treated with 0, 0.1, 0.5, 1 and 5 mg/ml RBE from both LTH and SHZ-2. LTH RBE exhibited a dose-dependent effect that was significantly different from SHZ-2 RBE for inhibition of *Salmonella* intracellular replication (Figure 1A). In MSIE cells, LTH RBE significantly inhibited ($p<0.05$) *Salmonella* replication at 0.5 and 1 mg/ml compared to vehicle control (Figure 1A), whereas in IPEC-J2, the LTH RBE significantly ($p<0.05$) inhibited at 5 mg/ml (Figure 1B). SHZ-2 RBE showed no inhibitory effects on *Salmonella* replication at any of the doses examined nor on any cell line. The doses of 1 mg/ml (MSIE cells) and 5 mg/ml (IPEC-J2 cells) were used in subsequent tissue culture experiments. The LTH and SHZ-2 RBE at 1 mg/ml (MSIE) and 5 mg/ml (IPEC-J2) were next evaluated for inhibition of *Salmonella* replication after 2, 4 and 6 h in MSIE and IPEC-J2. LTH and SHZ-2 RBE showed no differences in *Salmonella* replication between the time points after 2 h incubation. Thus, 2 h was chosen for comparison in subsequent experiments.

Effect of rice bran extracts on *Salmonella* invasion

To determine the effect of RBE on *Salmonella* invasion, MSIE cells were pre-incubated with RBE for 2 h before addition of GFP-labeled *Salmonella*. Fluorescence microscopy of GFP-labeled *Salmonella* revealed that LTH inhibited *Salmonella* invasion when compared to SHZ-2 (Figure 2A); however, quantification by percent infection revealed no significant differences in both the control and RBE treated MSIE cells (Figure 2B). RBE from LTH showed a 2-log cycle more inhibition of *Salmonella* invasion when compared to SHZ-2 (Figure 2C). These findings suggest that both RBE from LTH and SHZ-2 protected MSIE cells against *Salmonella* invasion, but the inhibition by the LTH RBE was of greater magnitude. Given that *Salmonella* can invade and colonize the pig, we next tested IPEC-J2 cells. LTH RBE inhibited *Salmonella* replication when compared to SHZ-2 RBE (Figure 2D); however, the inhibition was not statistically significant ($p>0.05$) as a percent of infection (Figure 2E). Only LTH RBE demonstrated inhibition ($p<0.05$) of *Salmonella* replication in IPEC-J2 cells as determined by CFU when compared to control and SHZ-2 RBE treatment (Figure 2F).

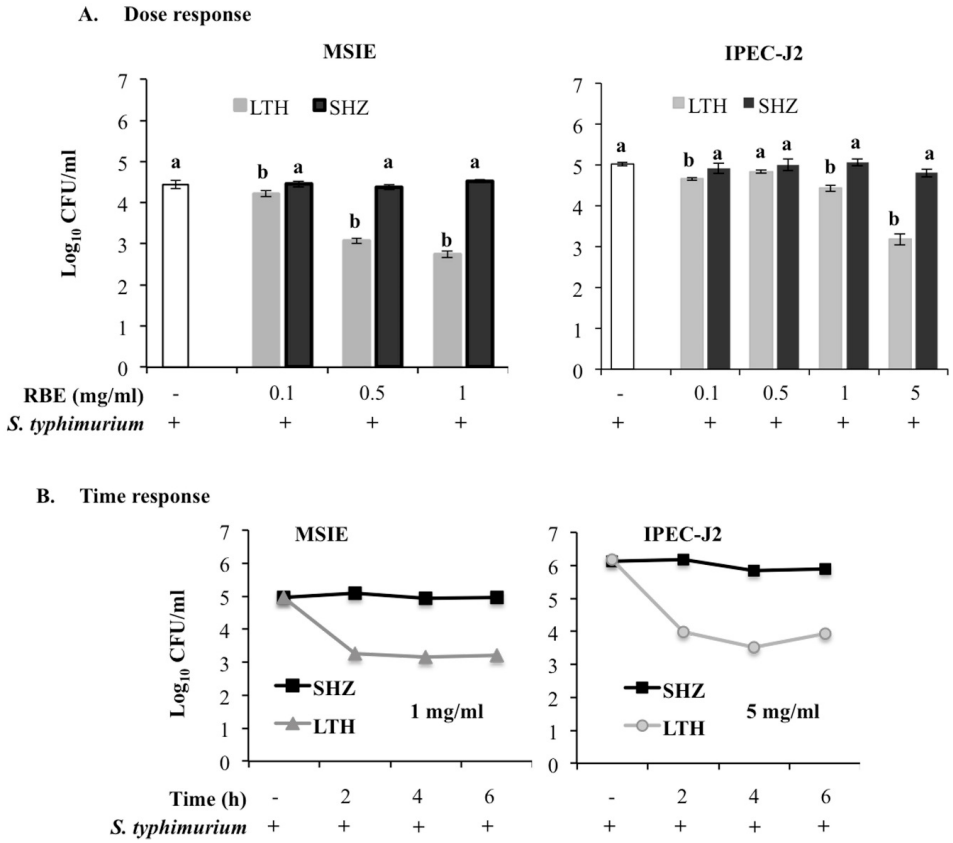
Differential effect of LTH and SHZ-2 bran extracts on *Salmonella* intracellular replication

We next quantified differences between the effects of LTH and SHZ-2 RBE (1 mg/ml for MSIE and 5 mg/ml for IPEC-J2) on *Salmonella* replication using fluorescence microscopy and culture based methods. Assessment of GFP-labeled *Salmonella* (Figure 3A) showed that LTH RBE inhibited *Salmonella* replication in MSIE cells to a greater extent than SHZ-2; yet, the inhibition was not statistically significant ($p>0.05$) when quantified as a percent of infection (Figure 3B). Inhibition of *Salmonella* replication in MSIE cells by LTH RBE, as measured by culture methods, was greater than 1.5 log cycles ($p<0.05$) when compared to control and SHZ-2 RBE treatment (Figure 3C). Similarly, LTH RBE appeared to inhibit *Salmonella* replication in IPEC-J2 more than SHZ-2 RBE by fluorescence microscopy (Figure 3D). The inhibition was not significant ($p>0.05$) when quantified as a percent of infection (Figure 3E). LTH RBE demonstrated a significant ($p<0.05$) inhibition of *Salmonella* replication in IPEC-J2 cells as determined by culture counts when compared to control and SHZ-2 RBE treatment (Figure 3F). *Salmonella* showed higher intracellular replication capacity in IPEC-J2 cells, but with similar infection efficiency (~25%) (Figure S2, right panel).

Metabolite profiling of LTH and SHZ-2 rice bran extracts

A non-targeted metabolomics analysis based on UPLC-MS/MS resulted in the identification of 429 metabolites in LTH and 407 metabolites in SHZ-2, including amino acids and their derivatives, carbohydrates, cofactors and vitamins, hormone metabolites, lipids, nucleotides, peptides, and secondary metabolites. Differences in chemical compositions between the two varieties were in lipids, secondary metabolites and dipeptides (Table 1).

There were 163 metabolites from LTH and 148 from SHZ-2 classified in the lipid biosynthesis metabolic pathway. Among lipids, galactolipids and phospholipids showed higher variation in the number of metabolites, as well as the relative abundance between LTH and SHZ-2 (Table 2). Figure 4A and Table S1 shows the range of metabolite expression for galactolipids, such as 1,2-dilinolenoyl-



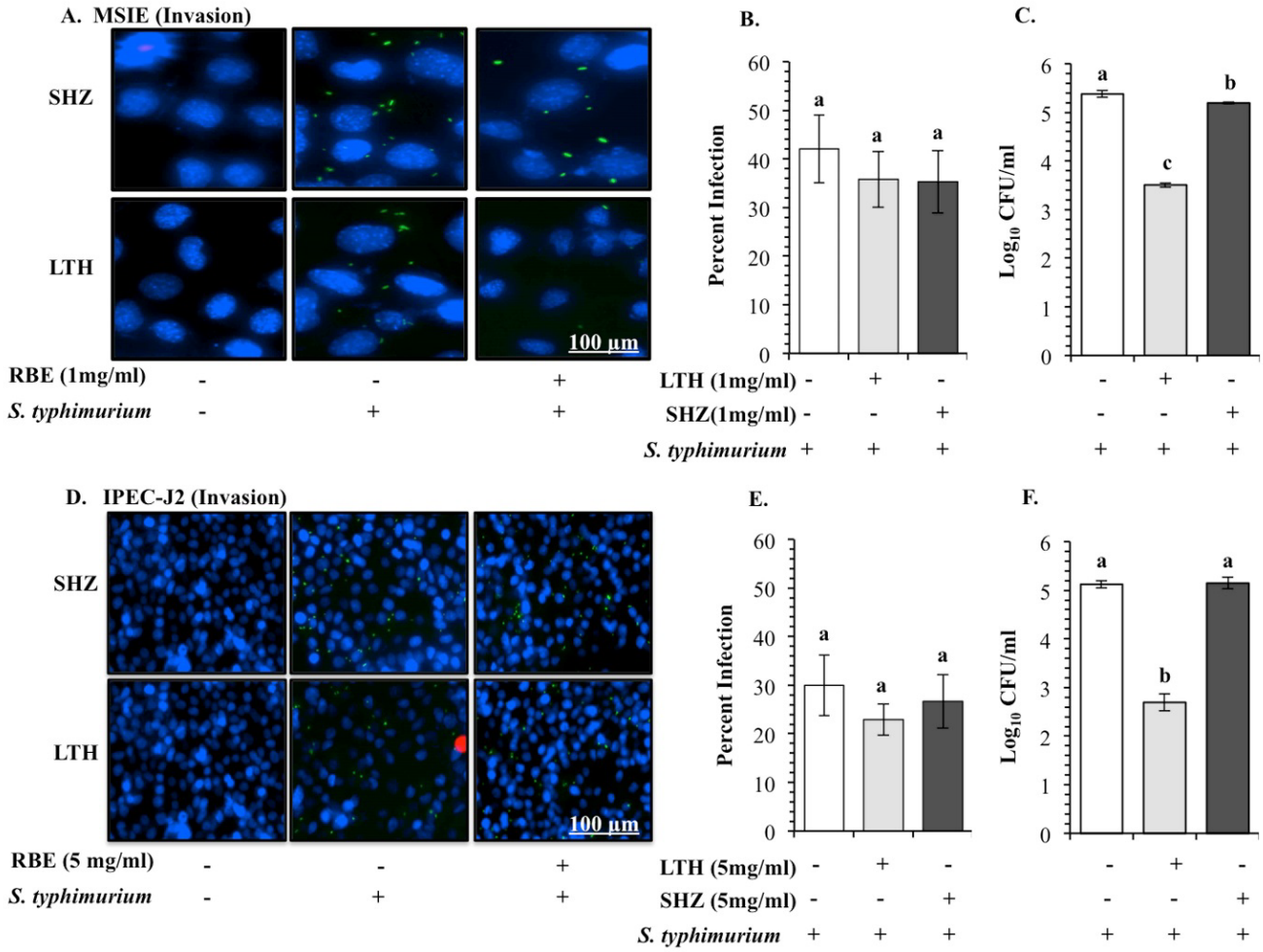
(A left panel) MSIE cells were treated with LTH and SHZ-2 RBE. (A right panel) IPEC-J2 cells were treated with LTH and SHZ-2 RBE. (B left panel) MSIE cells were treated with 1 mg/ml for 2, 4 and 6 h. (B right panel) IPEC-J2 cells were treated with 5 mg/ml for 2, 4 and 6 h. Significance was tested using one-way ANOVA and Dunnett's Multiple Comparison Test. Means ± S.E.M are presented as bars (n=3) representative of three independent experiments. Bars followed by the same letter are not significantly different ($p>0.05$).

Figure 1: Dose and time dependent effects of Rice Bran Extracts (RBE) on *Salmonella* replication in intestinal epithelial cells.

digalactosylglycerol (18:3/18:3), 1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3), 1,2-dilinoleoyl-galactosylglycerol (18:2/18:2) and 1-palmitoyl-2-linoleoyl-digalactosylglycerol (16:0/18:2) with higher fold difference in LTH. Figure 4B shows that the majority of phospholipid metabolites were also present in higher abundance and number for LTH when compared to SHZ-2. LTH showed a range of 5-87-fold increased expression in 23 metabolites from phospholipids (Table S1). The biochemicals within the galactolipid and phospholipid sub-metabolic pathways are mostly long-chain polyunsaturated fatty acids. Among peptide biosynthesis metabolic pathway, eight metabolites were detected in LTH and 11 metabolites in SHZ-2. SHZ-2 was higher in both relative abundances as well as number of metabolites detected for dipeptides (Figure 5A). Leucylglycine (5-fold), alanylleucine (8-fold), and valylglycine (9-fold) were higher in SHZ-2 as compare to LTH (Table S2). Under secondary metabolites, a total of 21 metabolites were identified from LTH and 16 from SHZ-2. The subsets of secondary metabolites included benzenoid biosynthesis, flavonoid biosynthesis, phenylpropanoid biosynthesis and terpenoid biosynthesis. The variations were observed in flavonoids and phenylpropanoids (Figure 5B and 5C). LTH had higher relative abundance and total number of metabolites in flavonoids compared with SHZ-2. Three flavonoids were absent in SHZ-2 (i.e., catechin, cyanidin glucoside and dihydroquercetin). Two other flavonoids (i.e., apigenin and chrysoeriol) were 32 and 6-fold higher, respectively, in SHZ-2, (Table S2). Although the total number of phenylpropanoids were higher in LTH, SHZ-2 had higher relative abundance in phenylpropanoids. Table S2 shows 2.5-fold difference increase in 4-hydroxycinnamate, ferulate and vanillate in SHZ-2 while p-coumaroylserotonin was almost 43 times higher in LTH.

Discussion

Dietary rice bran promotes gut mucosal immune responses against enteric pathogens [6,7,9], yet the mechanisms for how the effects differ among rice varieties and their biochemical composition is unknown. Herein we showed that LTH (japonica) significantly inhibits *Salmonella* invasion and intracellular replication in mouse and porcine intestinal epithelium to a greater extend when compared to SHZ-2 (indica). Although japonica and indica sub-species have the same origin, they were domesticated under different environmental conditions [24] and evidence supports variations in their seed metabalome profiles [25]. LTH and SHZ-2 were selected based on preliminary studies where they exhibited differences in inhibition of *Salmonella* colonization in mice [6,8,20], and because there is an available genetic population of recombinant inbred lines [29] that can facilitate subsequent identification of genes related to metabolite production [30]. Our



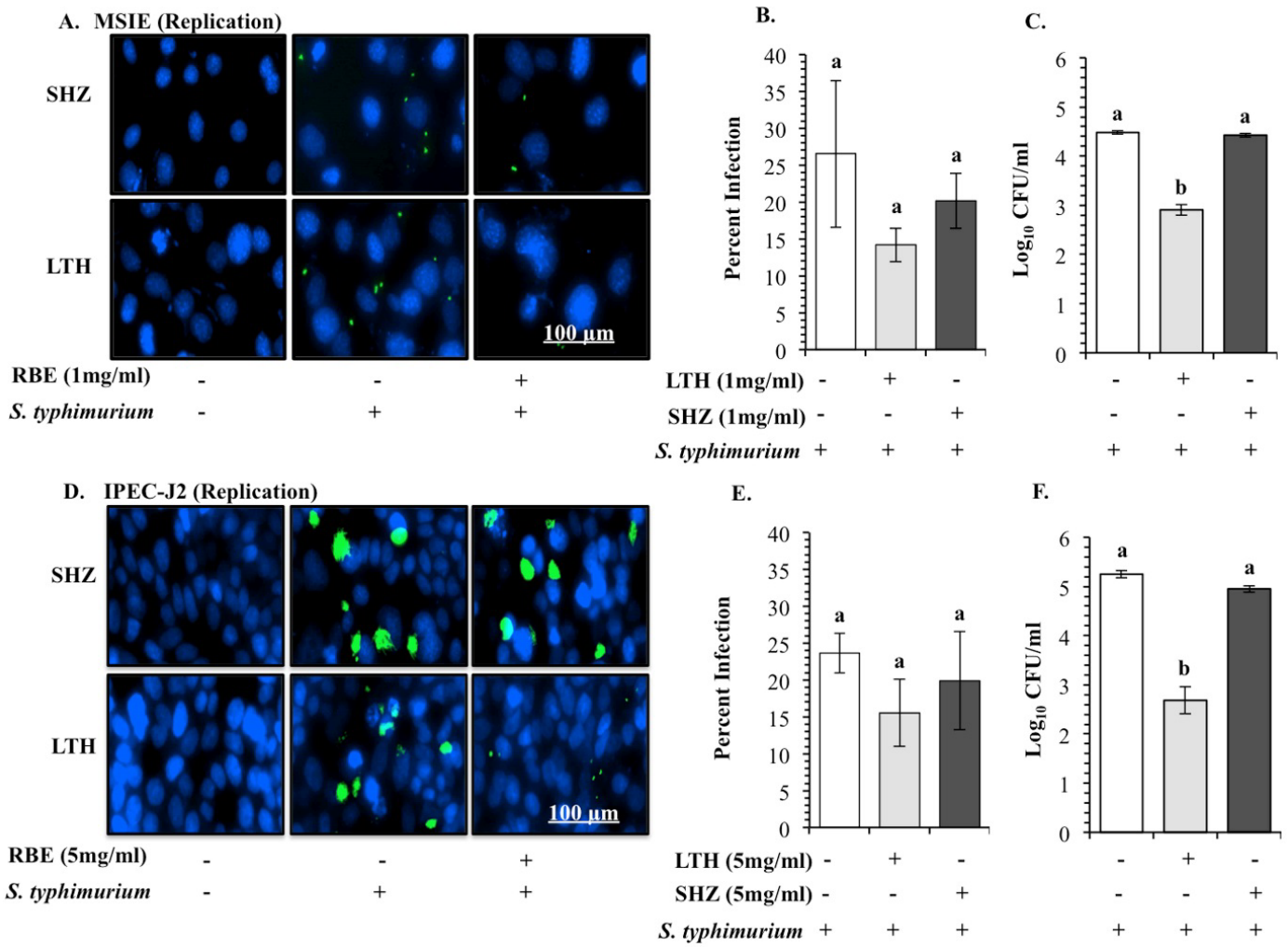
(A) MSIE cells were treated with or without 1 mg/ml of LTH or SHZ-2 for 2h before infection with or without *S. typhimurium* at 100 MOI for 1h and washed to remove extracellular *S. typhimurium*. Representative fluorescent images showing intracellular *S. typhimurium* (green) and nucleus (blue), images are representatives of three separate fluorescence infection experiments comprising at least three randomly selected fields of view per infection. (B) Quantification of total number of intracellular GFP *S. typhimurium*, determined from fluorescence images per infection, normalized per the MSIE cell count in field of view. *Salmonella* treatment to cells without bran extracts (white bars) was the positive control. LTH (gray bar) and SHZ-2 (black bar) extracts showed inhibitory effects on *Salmonella* invasion. (C) Viability of invasive *S. typhimurium* confirmed by CFU count on MacConkey agar, and expressed as log number of *Salmonella* CFU/ml. (D) IPEC-J2 cells were treated with or without 5 mg/ml of LTH or SHZ-2 for 2h before infection with or without *S. typhimurium* (100 MOI) for 1h. (E) Quantification of total number of intracellular GFP *S. typhimurium*, determined from fluorescence images per infection, normalized per the IPEC-J2 cell count in field of view. (F) Viability of invasive *S. typhimurium* confirmed by CFU count on MacConkey agar, and expressed as log number of *Salmonella* CFU/ml (panel F). Significance was tested using one-way ANOVA and Dunnett's Multiple Comparison Test. Means ± S.E.M are presented as bars (n=3) representative of three independent experiments. Bars followed by the same letter are not significantly different ($p>0.05$).

Figure 2: Effect of rice bran extracts on *Salmonella* Invasion.

findings indicate that most of the differences in LTH and SHZ-2 composition is secondary metabolites, lipids, and the dipeptides. We showed that the red-pigmented LTH rice variety has high levels of secondary metabolites (i.e., flavonoids), and lipids (galactolipids and phospholipids), which may be associated with inhibition of *Salmonella* invasion and intracellular replication. These findings demonstrate the potential of RBE as a dietary alternative for prevention of *Salmonella* infection.

Health properties of whole grains (including pigmented rice) have been widely reported [31], and many of which are related to secondary metabolites and fatty acids contents [32,33]. These compounds have effects on bacterial clearance and disease outcomes through various mechanisms [32,33]. The findings herein support that pigmented rice offers greater protection against enteric *Salmonella* infection as seen *in vivo* [8]. Flavonoids are classified as secondary metabolites, are abundant in many fruits and naturally occur in some cereals and legumes, and pose many beneficial health effects [34]. Flavonoids were shown to strongly inhibit arachidonic acid-derived inflammatory reactions, apoptosis, MAPK, and NF-κB pathways in macrophages [35]. An example, for a metabolite with higher abundance in LTH and among the flavonoids was catechin, which has been effective to reduce growth of *Staphylococcus aureus*, and *Salmonella Typhimurium* [36].

Studies have reported vegetable oils including rice bran oil have antimicrobial properties [15,37,38]. De Pablo et al. showed that host immunity and pathogen resistance may be influenced by the nutritional status [33]. Although, no single fatty acid is an answer for protection from food-borne pathogens (e.g., *Salmonella*), literature suggests that long chain polyunsaturated fatty acids could potentially



(A) MSIE cells were treated with or without 1 mg/ml of LTH or SHZ-2 for 2 h before infection with or without *S. typhimurium* (100 MOI) for 1 h and incubated for a further 24 h after removal of extracellular *S. typhimurium*. Representative fluorescent images showing intracellular *S. typhimurium* (green) and nucleus (blue), images are representatives of 3 separate fluorescence infection experiments comprising at least 3 randomly selected fields of view per infection. (B) Quantification of total number of intracellular GFP *S. typhimurium*, determined from fluorescence images per infection, normalized per the MSIE cell count in field of view. *Salmonella* treatment to cells without bran extracts (white bars) was the positive control. LTH (gray bar) and SHZ-2 (black bar) extracts showed inhibitory effects on *Salmonella* intracellular replication. (C) Viability of invasive *S. typhimurium* confirmed by CFU count on MacConkey agar, and expressed as log number of *Salmonella* CFU/ml. (D) IPEC-J2 cells were treated with or without 5 mg/ml of LTH or SHZ-2 for 2 h before infection with or without *S. typhimurium* (100 MOI) for 1 h. (E) Quantification of total number of intracellular GFP *S. typhimurium*, determined from fluorescence images per infection, normalized per the IPEC-J2 cell count in field of view. (F) Viability of invasive *S. typhimurium* confirmed by CFU count on MacConkey agar, and expressed as log number of *Salmonella* CFU/ml. Significance was tested using one-way ANOVA and Dunnett's Multiple Comparison Test. Means ± S.E.M are presented as bars (n=3) representative of three independent experiments. Bars followed by the same letter are not significantly different ($p > 0.05$).

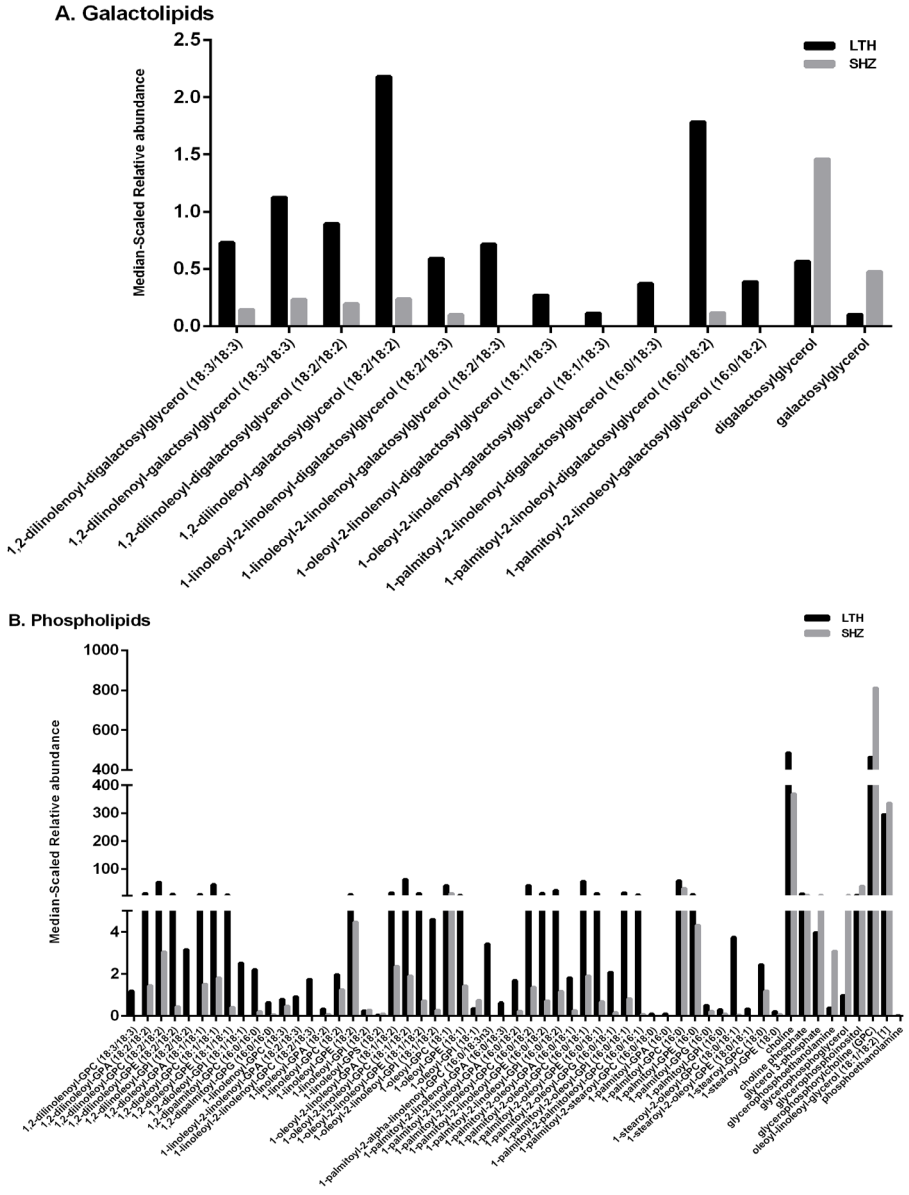
Figure 3: Effect of rice bran extracts on *S. typhimurium* intracellular replication.

Metabolic pathways	LTH	SHZ-2
Amino acids	117	115
Carbohydrates	53	51
Cofactors and vitamins	28	26
Hormone metabolism	4	4
*Lipids	163	148
Nucleotides	35	36
*Peptide	8	11
*Secondary metabolites	21	16
Total	429	407

Table 1: Number of rice bran metabolites annotated and clustered into metabolic pathways.

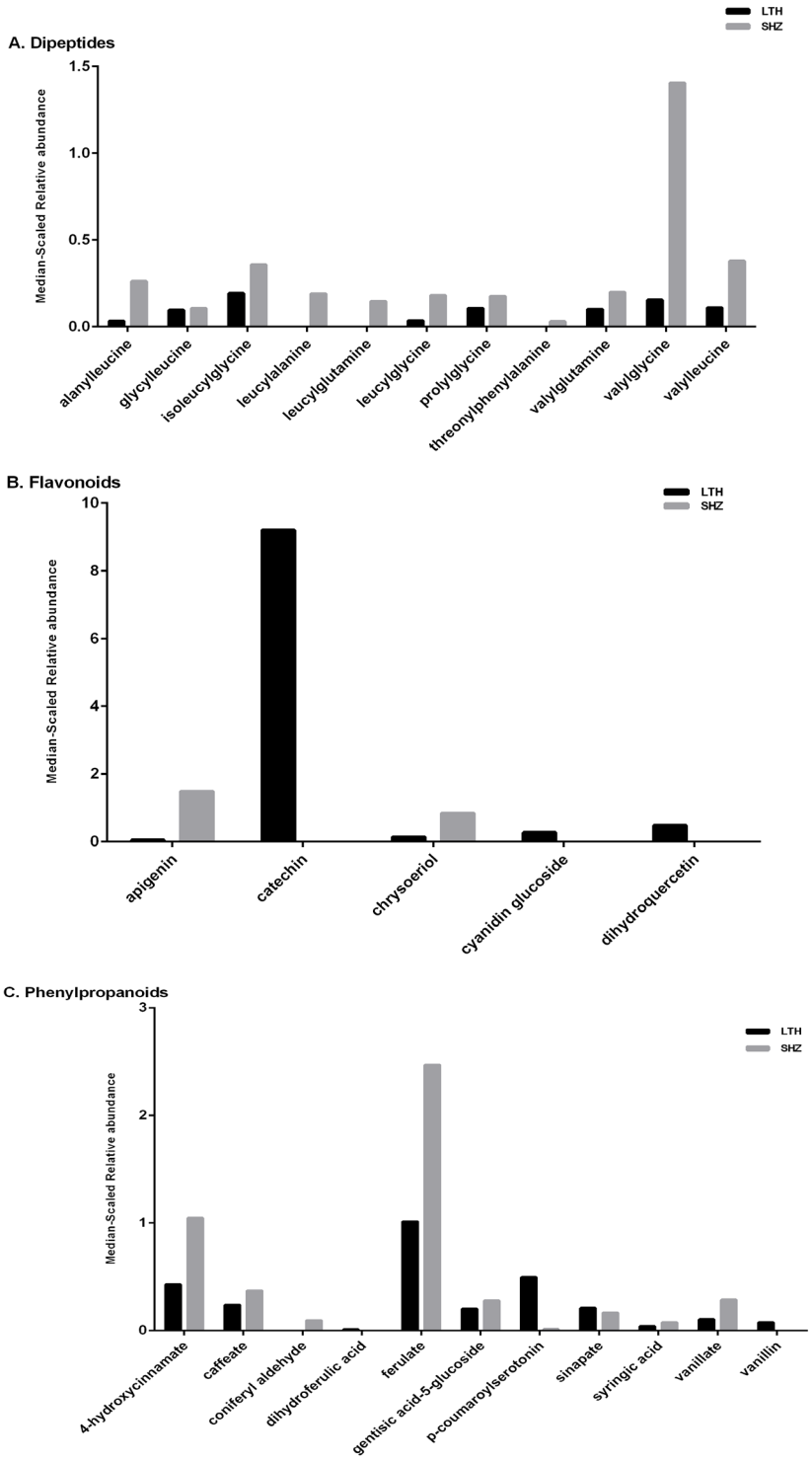
Sub-metabolic pathways	LTH	SHZ-2
Fatty acid amide	4	4
Fatty acid conjugate	1	1
Fatty acid ester	2	2
Fatty acid, Amino	0	1
Fatty acid, Dicarboxylate	11	11
Free fatty acid	31	31
*Galactolipids	13	8
Glycerolipids	33	33
Oxylipins	5	5
*Phospholipids	57	47
Sphingolipid	3	2
Sterols	3	3
Total	**163**	**148**

Table 2: Number of metabolites detected in sub-pathways of lipid metabolism.



Metabolites were normalized by median of relative abundance of entire dataset. Panel A shows the metabolites identified within the galactolipids sub-metabolic pathway and panel B shows those in the phospholipid sub-metabolic pathway.

Figure 4: Metabolome differences in LTH and SHZ-2 rice bran lipids.



Metabolites were normalized by median of relative abundance of entire dataset. Panel A indicated the metabolites identified within dipeptides sub-metabolic pathway (peptide), panel B represents flavonoids and panel C shows the metabolites identified in phenylpropanoids in LTH and SHZ-2.

Figure 5: Metabolome differences in LTH and SHZ-2 rice bran peptide and secondary metabolites.

alter the fate of intracellular bacterial burden based on their impact on the immune response, and therefore, fatty acids have to be properly titrated to avoid detrimental effects [39]. On the other hand, saturated fatty acids, including short-chain fatty acids, have either no effect or they have immune-enhancing and/or inflammatory effects, depending on the chain length [40-42]. For example, galactolipids such as 1,2-dilinolenoyl-digalactosylglycerol derived from Perilla frutescens was shown to inhibit superoxide generation [43,44]. We showed that SHZ-2 had higher levels of dipeptides compared to LTH. There are no reports to show that dipeptides promote enteric infections, but it is possible for dipeptides to supply essential amino acids required for bacterial growth [45]. While there are specific mechanisms for each metabolite, an emphasis is needed on emerging concepts for medicinal/aromatic plants to exhibit "phytochemical teamwork" or also referred to as bioactivity associated with a profile of plant metabolites [46].

We found that pre-treatment of intestinal epithelial cells with the LTH RBE reduced *Salmonella* invasion compared with the SHZ-2 RBE. This diminished invasion reduces the intracellular accumulation of *Salmonella* thereby suggesting that RBE may offer alternative preventive and/or treatment options. Follow up studies should examine rice bran effects on metabolic and receptor-mediumted mechanisms for protection against intracellular invasion of *Salmonella*.

Conclusion

Unique metabolite variation between LTH and SHZ-2 revealed rice bran components that may be responsible for blocking *Salmonella* invasion and intracellular replication. This study highlights a novel mechanism by which dietary rice bran can increase innate resistance against *Salmonella*. Multiple mechanisms for rice bran effects may involve synergies between a suite of bioactive rice bran components, the gut microbiome and the host cell. This metabolome investigation revealed a functional profile of rice bran components that merit continued research attention. Identification and characterization of metabolites from LTH rice bran may enhance our understanding of the relationship between rice genomics and bioactive bran metabolites. The set of metabolites identified that differ between rice bran varieties herein are candidates for protection against *Salmonella* infection, and may have important implications for reduced disease transmission.

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Author Contributions

IAG, JEL and EPR conceived and designed the study. AM provided bran of the rice varieties. IAG, JOM, JRW performed the chemical and biological analyses. IZ, IAG and CB performed metabolomics analysis. IAG, JOM, IZ and EPR interpreted the results and wrote the manuscript. All authors made editorial comments, read and approved the final version of the article.

Conflict of Interests

The authors declare no conflict of interest.

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