Autophagy Genes of Host Responds to Disruption of Gut Microbial Community by Antibiotics

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Abstract

Background Defective autophagic machinery, such as that in Crohn’s disease patients homozygous for ATG16L1 risk allele, is associated with alteration of resident gut bacterial communities. However, whether or not host autophagy responds to changes in the resident gut microbial community is not known. Here, we investigated the effect of antibiotic-induced disruption of the gut microbiome (dysbiosis) on autophagy gene expression and the expression of antimicrobial peptides/protein (AMP) over time.

Aim To test the hypothesis that antibiotic treatment may cause time-dependent changes in gut bacterial density, autophagy genes, and antimicrobial protein/peptide gene expression.

Methods Mice (n = 8 per group) were treated with antibiotic cocktail and sacrificed at different intervals of recovery (days 3, 7, 10, 14, 21, 28, 35, and 42) post-antibiotics. DNA and RNA were extracted from small intestinal tissues. Bacterial density, expression of host autophagy genes, and AMP genes were analyzed by relative quantitative PCR. Fold change difference in comparison with untreated control group was calculated using 2−ΔΔCt method. Statistical analysis was performed using nonparametric Mann–Whitney test.

Results Gut bacterial density changed in a time-dependent fashion in response to antibiotic treatment. These changes were concurrent with upregulation of autophagy genes and antimicrobial peptide/protein gene expression. We further showed that an oral gavage of a resident microbe Desulfovibrio, which bloomed in antibiotic-treated animals, induced Atg5 and lysozyme (Lyz) gene expression.

Conclusion Autophagy genes respond to dysbiosis induced by antibiotics. This response may be a host mechanism to detect and possibly correct dysbiosis by activating antimicrobial peptides/proteins that control the microbial load in the gut.

Keywords Antibiotics · Desulfovibrio · Autophagy · Antimicrobial peptide/protein

Introduction

Autophagy is a host innate immunity pathway responsible for maintaining cellular homeostasis. One of the many functions of autophagy is elimination of intracellular pathogens. Selective elimination of intracellular pathogens within an autophagosome may occur following induction of autophagy by either free microorganisms in the cytosol, e.g., S. pyogenes or sequestered microorganisms in phagosomes, e.g., M. tuberculosis. By avoiding autophagy, some microorganisms are able to achieve a persistent state of infection, e.g., L. monocytogenes. In the intestine, autophagy has been reported to play a protective role against intracellular pathogens such as Salmonella [1], Shigella [2], Listeria [3], Campylobacter [4], Vibrio cholerae [5], and adherent invasive E. coli [6]. While abundant
evidence supports the role of autophagy in controlling intracellular pathogenic microbes, very little is known about its potential role in responding to or controlling non-pathogenic, extracellular resident gut microbes. A study by Sadaghian et al. [7] showed that there were an increased number of gram-negative gut microbes in the intestinal lumen belonging to the families Enterobacteriaceae, Bacteroidaceae, and Fusobacteriaceae in Crohn’s disease patients homozygous for the ATG16L1 T300A risk allele. This suggests that disruption of autophagy is associated with altered resident gut microbial community. However, the converse whether disruption of gut bacterial community is associated with changes in autophagic machinery is not known. Additionally, in autophagy-deficient mice and in Crohn’s disease patients with ATG16L1 risk allele, the distribution of antimicrobial peptide and protein (AMP) was reported to be abnormal in intestinal Paneth cells [8, 9]. AMPs are ancient immune defense mechanisms that play a crucial role in regulating the resident gut microbes [10]. These findings suggest the possibility that altered AMP expression or secretion associated with defective autophagic machinery might result in the disruption of resident microbial community of the gut or dysbiosis as characterized by qualitative and quantitative changes in the gut microbial composition, distribution, and metabolic activity. A number of environmental factors may cause microbial dysbiosis in the gut including antibiotics [11, 12]. Several studies have demonstrated that antibiotic treatment causes short-term and long-term disruption in the gut microbial community [13–16]. Antibiotics reduce the density of some members such as Firmicutes while augmenting the density of others such as Proteobacteria and Enterobacteriaceae. It has been shown that hydrogen sulfide (H$_2$S) produced by sulfate-reducing bacteria (SRB) such as Desulfovibrio spp. belonging to the phylum Proteobacteria confers selective advantage to these bacteria in the presence of antibiotics to favor their survival during antibiotic treatment [15, 16].

In this study, we used antibiotics to disrupt the gut microbial community in mice and followed longitudinally over 42 days the dynamics of gene expression of autophagy proteins as well as that of antimicrobial peptides/proteins (AMP).

Materials and Methods

Animals

Five-week-old, female C57BL/6 mice (20–25 g) were utilized in the study. Mice were purchased from the Charles River Lab (Wilmington, DE). Upon arrival, animals were housed in groups of three in polypropylene cages and placed on a 12-h light/dark cycle and kept on a standard rodent diet (Harlan Teklad Laboratory Diets). Mice were subject to one-week acclimatization period.

Study Approval

The procedures were approved by the Institutional Animal Care and Use Committee at the New Mexico VA Health Care System following guidelines provided by the Guide for the Care and Use of Animals of the National Research Council of the National Academies.

Antibiotic Treatment

All the antibiotics were purchased from Sigma-Aldrich. Seventy-two mice were divided into nine groups. Group 1: Controls mice not treated with antibiotics. Group 2–9: Mice treated with an antibiotic cocktail as described [17]. Briefly, mice were administered a cocktail of antibiotics consisting of colistin (C4461) (4.2 mg/kg), gentamicin (G4918) (3.5 mg/kg), kanamycin (K1876) (40 mg/kg), metronidazole (M1547) (21.5 mg/kg), and vancomycin (94747) (4.5 mg/kg) in their drinking water ad libitum for 3 days. All antibiotic-treated mice were then given regular water for 2 days followed by a single dose of clindamycin (C6427) (10 mg/kg) (i.p.). Control mice received an i.p. injection of phosphate buffered saline (PBS). Mice were allowed to recover for up to 6 weeks post-treatment before euthanasia for sample collection. Mice were euthanized at various intervals, group 1: day 7, group 2: day 3, group 3: day 7, group 4: day 10, group 5: day 14, group 6: day 21, group 7: day 28, group 8: day 35 and group 9: day 42. Small intestinal tissue samples corresponding to proximal and distal most one-third regions of the small intestine were collected and stored in RNA later (Thermo Fisher Scientific AM7021) and at −80°C for further analysis.

Quantitative PCR

DNA (DNeasy Blood & Tissue Kit, Qiagen: 69506) and RNA (RNeasy Mini Kit, Qiagen: 74106) were extracted from collected intestinal tissues. cDNA synthesis was carried out using cDNA kit (Thermo Fisher Scientific: 18080-051). Real-time quantitative PCR was performed using SYBR green (Qiagen: 204145). Bacterial density of total universal 16S rRNA gene, Desulfovibrio, Bacteroidetes, and Firmicutes was analyzed in the DNA samples (100 ng) isolated from the tissues. 16S rRNA gene values were normalized against the host 18s rRNA gene and the individual member profile was normalized against universal 16S rRNA gene profile. Host autphagic and AMP gene expression was analyzed in the cDNA (100 ng) isolated from RNA from intestinal tissues. All the primer sequences are described.
Primer sequences for real-time PCR

| Universal 16S F | ACTCTACGGGAAGGCACGAG |
| Universal 16S R | ATTACCCGCGCTTGGG |
| Firmicutes F | GGAGYATGTGGTTTAATTCGAC |
| Firmicutes R | AGCTGACGACAACCATGCA |
| Bacteroidetes F | GTACTGACGACAACCATGCA |
| Bacteroidetes R | ATTACCCGCGCTTGGG |
| DSV F | CCGTAGATATCTGGAGGAACATCAG |
| DSV R | ACATCTAGCATCCATCGTTTACAGC |
| 18s F | GTAACCCTTGAAACCCCAT |
| 18s R | CCATCCAATCGGTAGTAGCG |
| Atg5 F | ATATGAAGGCACACCCCTGA |
| Atg5 R | CCAAGGAAGAGCTGAACTTGA |
| Atg16 F | AGGCGTTCGAGGAGATCATT |
| Atg16 R | TTCTGCTTGTAGTTTCTGGGTCA |
| Irgm1 F | GAGACTGTGGCAACATTG |
| Irgm1 R | CCGATGACTCGAAGTGCATTG |
| Lyc F | GGATCAATTGCAGTGCTCTG |
| Lyc R | CAGTCCGAAATATACGGGAC |
| RegIII γ F | GTATGATGCAGATATGGCCTG |
| RegIII γ R | ATATTGGCCACTGTTACCAC |
| Crypt 4 F | GACCAGAAGGATCTGCTT |
| Crypt 4 R | GCATATCAGATCTGCTGACGA |

Bacterial Culture

The sulfate-reducing bacterium, *Desulfovibrio vulgaris* Hildenborough NCBI 8303, was grown on a lactate–sulfate medium according to previously published methods [18]. *D. vulgaris* was gavaged in mice as described previously [19]. *Bacteroides thetaiotaomicron* VPI-5482 was grown on BHI medium (Sigma-Aldrich). Bacteria were enumerated by counting with Petroff–Hausser counting chamber (Hausser Scientific), and approximately $1 \times 10^9$ bacteria were gavaged into the stomach via the mouth per mouse for each experiment.

Cell Culture and Treatments

RAW 264.7 and IEC-6 cells were purchased from ATCC protocol. RAW cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 units/ml of penicillin and 100 μg/ml of streptomycin. IEC-6 cells were grown in DMEM supplemented with 10% fetal bovine serum and 0.1 unit/ml of bovine insulin. Cells were treated for 72 h with the cocktail of antibiotics. See above. Rapamycin (Sigma: R8781) was added at a concentration of 50 or 100 nM for 4 h. Cells or tissues were lysed in Lysis buffer (Thermo Fisher Scientific: 87787) containing protease and phosphatase inhibitors (Thermo Fisher Scientific: 1861281) and were processed for Western blot.

Western Blot

50 μg samples were run on SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk in PBS-Tween (0.1%) for 30 min followed by overnight incubation in lysozyme antibody (Abcam: ab108508), actin antibody (Cell Signaling: 4970) or LC3 antibody (Cell Signaling: 27755). Blots were washed in 0.1% PBS-tween and incubated with secondary antibodies (Cell Signaling: 7074) at room temperature for 1 h. Blots were developed using the enhance chemiluminescence (Thermo Fisher Scientific: 32106).

Statistical Analysis

All graphs were generated using GraphPad Prism 5. For qPCR data analysis, we were assisted by a biostatistician, Clifford Qualls, PhD. A nonparametric Wilcoxon–Mann–Whitney test was used to determine the statistical significance of changes between groups. *P* values $< 0.05$ were considered significant.

Results

Antibiotic Treatment Induces Small Intestinal Bacterial Overgrowth

To study the potential involvement of autophagic machinery as a host response mechanism to disruption of the gut microbial community, we disrupted the mouse resident gut microbiome with antibiotics [17] and analyzed expression of autophagy and antimicrobial peptide genes. Mice were allowed to recover for up to 42 days after cessation of treatment. Small intestinal samples were collected following euthanasia on days 3, 7, 10, 14, 21, 28, 35, and 42. Since the normal small intestine has a much lower microbial density and tighter regulation of gut microbes compared to the large intestine, we expected the impact of disruption of gut microbial community by antibiotics to be dramatic in this region.

We tested the effect of antibiotic treatment on mucosa-associated bacteria in the small intestine. The bacterial load in the proximal (corresponding to duodenum and proximal jejunum) and distal one-third regions (corresponding to ileum) of small intestine was analyzed using quantitative PCR (qPCR) with a universal 16S rRNA gene primer and genus-/phyla-specific primers targeting *Desulfovibrio*, Bacteroidetes, and Firmicutes.
By day 42, there was a ~tenfold increase in the density of universal 16S rRNA gene in both proximal and distal regions ($P$ value < 0.01) (Fig. 1a, b; Table S1) demonstrating dysbiosis in the form of small intestinal bacterial overgrowth. A robust change also occurred in the density of Desulfovibrio (DSV) following antibiotic treatment. DSV load increased sharply by day 7, ~600-fold increase in proximal ($P$ value < 0.01), and ~1500-fold in distal region ($P$ value < 0.01) (Fig. 1c, d; Table S1) and remained significantly higher than controls on days 10, 14, 21, 28, and 35. However, on day 42, DSV levels dropped drastically with no significant difference seen compared to controls ($P$ value > 0.05). This pattern was observed in both the proximal and distal regions of the small intestine. Similar to DSV, a common phylum of the gut microbial community, Bacteroidetes, also exhibited dramatic density shifts in the proximal and distal region of the small intestine during the recovery from antibiotic treatment (Fig. 1e, f; Table S1). However, unlike DSV, two peaks of Bacteroidetes density were observed: the first on day 7, ~400-fold increase in proximal ($P$ value < 0.01) and ~100-fold in distal region ($P$ value < 0.01) and the second on day 42, ~750-fold increase in proximal ($P$ value < 0.01) and ~350-fold in distal region ($P$ value < 0.01). Firmicutes dropped down significantly lower than the controls on day 42 ($P$ value < 0.01) (Fig. 1g, h; Table S1). Thus, antibiotic treatment led to small intestinal bacterial overgrowth and a change in density of individual members of the gut bacterial community.

**Dysbiosis Is Concurrent with Changes in Autophagy Gene Expression**

To examine whether autophagy genes of the host are responsive to changes in gut microbial community, we followed Atg5, Atg16l1 and Irgm1 by quantitative PCR (qPCR) in the same tissue samples that were examined for bacterial density. We found that Atg5 and Atg16 expression increased in response to antibiotic-induced dysbiosis when compared to controls. The first dramatic change in Atg5 expression occurred on day 10 in both the proximal (~100-fold increase; $P$ value < 0.01) and distal (~35-fold increase; $P$ value < 0.05) regions of the small intestine (Fig. 2a, b; Table S1). While the levels of Atg5 in the proximal region remained significantly higher than the control until day 42, its levels in the distal region showed more fluctuations, dropping down on days 14, 21, and 28 (non-significant compared to the control) and rising again on days 35 and 42. Changes in Atg16 were modest in comparison with Atg5 in the proximal small intestine ($P$ value < 0.01), but were comparable to Atg5 in the distal region ($P$ value < 0.05) (Fig. 2c, d; Table S1). There was a significant correlation between Atg5 and Atg16 expression (Figure S1) consistent with their roles as parts of the complex required for autophagosome formation. A less robust response was observed in Irgm1 gene expression which showed significantly higher values only on day 42 compared to controls in the proximal region (~sixfold increase compared to control; $P$ value < 0.05) and in the distal region on day 42 and day 35 (~3.5-fold increase; $P$ value < 0.05) (Fig. 2e, f; Table S1). Thus, disruption of resident gut bacteria corresponded with longitudinal changes in the genes of autophagic machinery. In addition, we observed an increase in Atg16L1 protein in most antibiotic samples when compared to control tissue samples in both the proximal (Fig. 2g) and distal region of small intestinal tissues (Fig. 2h).

**Antibiotics Alone Do Not Directly Induce Autophagy**

To rule out the possibility that antibiotics directly increased autophagy, we treated RAW 264.7 macrophages as well as IEC-6, small intestinal epithelial cells with the same antibiotic cocktail as the one used in animal study. Antibiotic treatment did not induce increase in LC3-II expression, marker of autophagy induction, in IEC-6 cells (Fig. 3a, b) or in RAW cells (Fig. 3c, d) when treated with antibiotics for 3 days, the same duration as that of treatment in mice. In contrast, treatment with rapamycin triggered robust autophagic response in both the cell lines. Moreover, the effect of antibiotic treatment on autophagic machinery in vivo was observed weeks after antibiotic cessation, indicating that the changes observed in the autophagy gene expression in mice were not a direct effect of antibiotics on host autophagy.

**Antimicrobial Peptide/Protein (AMP) Gene Expression Is Concurrent with Changes in Autophagy Gene Expression**

We hypothesized that increase in expression of autophagy genes may be associated with an increase in AMP gene expression. This is based on previous reports that normal lysozyme distribution depended on functional autophagy as there was a defect in both the expression and intracellular distribution of lysozyme in Paneth cells in the setting of non-functional autophagy. In our study, we observed robust changes in the expression of the lysozyme gene Lyz (Fig. 4a, b; Table S1). Starting day 7, a significant increase in Lyz expression was observed in the proximal small intestine ($P$ value < 0.05) which remained consistently higher than the control with maximum levels reached on day 42 (~700-fold higher than control; $P$ value < 0.01). In the distal region, significantly higher values were detected starting day 7, reaching maximum on day 42 (~7000-fold
increase; P value < 0.001). We observed a strong correlation between *Atg5* and *Lyz* gene expression (Figure S2) suggesting their positive relationship. This is in agreement with the previous studies in Paneth cells. We also analyzed antimicrobial peptides *RegIIIγ* (Fig. 4c, d; Table S1) and *Cryptdin4* (*Crypt 4*) (Fig. 4e, f; Table S1). In the proximal and distal regions, both *Reg IIIγ* and *Crypt 4* showed significant increase in expression on day 35 and day 42.
Longitudinal changes were observed in host autophagy genes during the recovery phase of antibiotic treatment. Proximal and distal one-third region of small intestinal tissues were collected on different days post-antibiotic treatment. Control samples were collected on day 7. RNA was isolated from tissue samples and cDNA was synthesized. qPCR was carried out using primers specific for Atg5 (a, b), Atg16 (c, d), and Irgm1 (e, f). Data are presented as median value with the 25th and 75th quartiles in each box plot. The whiskers represent highest and lowest data points. ***P < 0.001; **P < 0.01; *P < 0.05 (Mann–Whitney U test). g, h Proteins were extracted from small intestinal proximal and distal tissue samples from control and antibiotic-treated (day 28 and day 42 shown) mice with lysis buffer and 50 μg was used for Western blotting for probing for Atg16L1 and actin. Atg16 protein levels were measured using ImageJ software as a relative density.
Atg5 and Lyz Genes Are Also Amplified in Response to Gut Bacterium Desulfovibrio vulgaris

Antibiotic treatment in our animals led to a bloom in the bacteria of the genus Desulfovibrio (DSV) (Fig. 1c, d) that are normal residents of the gut. DSV belongs to a group of sulfate-reducing bacteria (SRB) which are increased in number in the stool of patients with ulcerative colitis [21]. The presence of increased load of DSV in the small intestine may act as a potential direct or indirect signal to trigger autophagic machinery as the host response to detect and possibly correct the disruption of gut microbial community by activating AMPs such as lysozyme. We tested this possibility by administering DSV species D. vulgaris by oral gavage and analyzed Atg5 and Lyz gene expression in proximal and distal regions of the small intestine. We found that a single oral administration of D. vulgaris caused a significant increase in both Atg5 and Lyz gene expression to a similar magnitude (Fig. 5a–d) confirming our observations in the animals treated with antibiotics. We also observed corresponding increase in the protein expression of lysozyme in animals gavaged with D. vulgaris compared to control animals (Fig. 5e). Atg5 in the distal region did not respond as well when compared to its expression in the proximal region. This is consistent with our antibiotic treatment data where proximal Atg5 expression was more robust. This finding provides a more direct evidence of the relationship between resident gut bacteria and autophagy genes in our study. In contrast to DSV, when we gavaged the mice with B. thetaiotaomicron, bacteria that belong to one of the most predominant phyla in the gut, Bacteroidetes, we did not observe the same robust effects on Atg5 or Lyz compared to DSV (Fig. 5g–i). Thus, increase in autophagy genes is not a universal phenomenon in response to all the gut bacteria and maybe specific to those that accumulate in adverse conditions. Overall, our results suggest that changes in the gut bacterial community in the small intestine trigger autophagic machinery and AMPs of the host. This response may serve as a potential host mechanism to control dysbiosis such as bacterial overgrowth possibly using AMPs as the downstream effectors of autophagy.

Discussion

Our data demonstrate for the first time changes in host autophagy genes (Atg5, Atg16l1 and Irgm1) and AMP genes (Lyz, Reg IIIc and Crypt 4) in mice recovering from antibiotic treatment in a time-dependent fashion.

The antibiotic cocktail used in our study induced gut dysbiosis as represented by small intestinal bacterial overgrowth (SIBO). Antibiotics have been widely reported to induce dysbiosis causing long- and short-term changes in the gut microbial community [21–25]. While antibiotics are disruptors of the gut microbiome, certain antibiotics such as rifaximin may be used to treat dysbiosis as shown in randomized, placebo-controlled trials where poorly absorbable antibiotic significantly reduced symptoms in patients with conditions associated with gut dysbiosis [26–29].
While the protective role of autophagy in clearing intracellular pathogens is well established, whether or not autophagy has a role in maintaining homeostasis of extracellular, non-pathogenic resident gut microbes is not known. As changes in autophagy genes were associated with alterations in extracellular, non-pathogenic resident gut bacterial community, our findings advance our understanding of autophagy and bacteria by extending its role beyond the control of intracellular pathogens.

We showed that antibiotic treatment resulted in marked small intestinal bacterial overgrowth as the density of the total 16S rRNA gene increased ~tenfold by day 42. An even more impressive change was observed in individual members of the bacterial community. Specifically, there was an increase in the Bacteroidetes/Firmicutes ratio, consistent with previous studies [30]. In addition, we observed dramatic longitudinal shifts in the density of genus Desulfovibrio (SRB).

Corresponding to the shift in gut bacterial density and composition, the expression of autophagy and AMP genes changed longitudinally. Significant increase in the expression of Atg5 and Atg16 occurred concurrently with increased expression of AMP genes such as Lyz, RegIIIγ and Crypt4, all of which are known to play a crucial role in the maintenance of the homeostasis of the intestinal microbiome.

The order of events was revealing as a change in the gut bacterial density occurred prior to a surge in expression of...
**Fig. 5** *D. vulgaris* induced expression of genes *Atg5* and *Lyz* in mice. Number of mice per group = 6. Control animals were gavaged with saline and, *D. vulgaris* gavaged animals were administered approximately 10^9^ bacteria. Mice were euthanized after 45 min post-gavage and tissue samples collected from proximal (a, c) and distal (b, d) one-third region of the small intestine. cDNA was synthesized and qPCR was performed using gene targeted primers. Fold change values are calculated using 2^-\Delta\Delta C_T^ method and were plotted as log base 2 for *Atg5* (a, b) and log base 10 for *Lyz* (c, d). Tissue samples from *D. vulgaris* treated or control mice (N = 2) were subjected to Western blot analysis (e) and probed for lysozyme protein. Actin was used as a loading control. Mice (N = 6) were also gavaged for 45 min with another gut bacterium *B. thetaiotaomicron* and small intestinal one-third proximal (f, h) and distal tissues (g, i) were analyzed for *ATG5* and *LYZ* expression. Data are presented as median value with the 25th and 75th quartiles in each box plot. The whiskers represent highest and lowest data points. Two-tailed Mann–Whitney *U* test was performed for statistical analysis. *P* values < 0.05 were considered significant. **P** < 0.01; *P* < 0.05
these host genes, suggesting that alteration of the gut microbial community may signal host autophagy and AMP machinery. We provided more direct evidence that the correct sequence is microbes then autophagy and AMP genes by testing the effects of a single oral gavage of *D. vulgaris* on autophagy and AMP genes in the small intestine. This species of microbe was selected since *Desulfovibrio* genus was found to bloom in density in our study as an early response to antibiotic treatment.

*Desulfovibrio* is a member of sulfate-reducing bacteria (SRB) that produces hydrogen sulfide (H$_2$S) as a byproduct of their metabolism. While the carriage rate of SRB varies among different human populations [31], *Desulfovibrio* was uniformly present in our animals. It is known that H$_2$S may confer a survival advantage to SRB under adverse conditions such as exposure to antibiotics [15]. H$_2$S has also been shown to induce [32, 33] as well as inhibit autophagy [34, 35] in various studies. An overgrowth of SRB has been found in inflammatory disorders such as UC [20, 36] and periodontitis [37]. Our data would suggest that growth of SRB is also favored by antibiotic treatment.

Our group has previously shown that introduction of *Desulfovibrio vulgaris* (DSV) by oral administration causes a transient increase in concentration of these bacteria in the small intestine and in cecum [19] sufficient to cause direct effects on the physiology of the host. In the present study, we found that oral administration of *D. vulgaris* treatment caused an increase in *Atg5* and *Lyz* in a manner similar to that seen in antibiotic-treated mice. Thus, perturbation of the gut microbial community either broadly by antibiotics or specifically by administering a single member of gut bacterial species (one that is known to survive and thrive under adverse conditions) triggers the genes of autophagic machinery and AMP. This response may serve as a potential mechanism for detecting and regulating the abnormal changes in the gut microbial load seen in dysbiosis. A strong correlation between *Atg5* and *Lyz* gene supports their positive relationship which is in agreement with previous studies [8, 38].

How autophagic genes are triggered in the setting of dysbiosis induced by antibiotic is not known. Interestingly, hydrogen sulfide produced by SRB such as DSV has been shown to induce autophagy in some studies. Moreover, it is known that LPS produced by gram-negative bacteria induces autophagy and DSV also produces LPS. Thus, a bloom in DSV in the small intestine resulting in an increase in the signaling molecules such as LPS or H$_2$S could provide an underlying mechanism for inducing autophagy genes in the setting of bacterial overgrowth.

Published studies provide clues that *Bacteroides* spp. may be associated with autophagy. Polysaccharide A (PSA) produced by *Bacteroides fragilis* induces anti-inflammatory IL-10, suppresses pro-inflammatory IL-17, and prevents gut inflammation in a chemically induced model of colitis [39, 40]. PSA is a ligand for toll-like receptor 2 (TLR2) which is involved in the induction of autophagy [32, 41]. The anti-inflammatory role of PSA suggests that it may be one of the critical microbial factors in maintaining gut homeostasis. Also, mono-colonization of germ free mice with *B. thetaiotaomicron* was found to induce expression of antimicrobial protein angiogenin (Ang4) [42] and RegIIIγ [43]. In contrast, in IBD patients and in experimental colitis model with lower levels of vitamin D receptor (VDR), a decreased ATG16L1 and lysozyme expression in the intestine was observed which was found to be associated with higher level of *Bacteroides fragilis* [44]. Also, *B. fragilis* has also been found to be associated with IBD [45]. In our study, we did not observe an increase in *Atg5* and *Lyz* at the time point tested when we gavaged mice with *B. thetaiotaomicron*, suggesting that *Bacteroides* alone may not induce autophagy and intermediary factors such as VDR may be required.

We found that starting day 7, there was a significant increase in *Lyz* expression in the small intestine with maximum levels reached on day 42. Correspondingly, the density of *Desulfovibrio* returned to baseline over that time period. Thus, one or more changes in microbial community may trigger autophagic machinery, which in turn may promote the production of AMP to address the dysbiosis and suppress the overgrowth of *Desulfovibrio* and perhaps other bacteria. This response of the host would then allow the density of *Desulfovibrio* to return to baseline by day 42. Lysozyme has been previously shown to inhibit DSV culture [46]. Our data would suggest that gut microbial signals, autophagic machinery and AMP are closely linked and their interplay is crucial to the response of the host to the dysbiosis triggered by antibiotic treatment. By means of the generation of lysozyme and other AMPs, autophagy may act extracellularly to control abnormal growth of resident microbes in the setting of dysbiosis.

Our study has limitations. We did not demonstrate a direct induction of autophagy such as increase in LC3-II by Western blots in our tissue samples. Instead, the increase in atg genes in our study showed that autophagy gene was induced in the setting of antibiotic-induced dysbiosis. However, several studies have shown that an increase in autophagy genes is accompanied by induction of autophagy [47–50]. Thus, increase in expression of autophagy genes in our study may lead to or occur concurrently with induction of autophagy. Further study is needed to demonstrate autophagy in response to antibiotic-induced small intestinal dysbiosis.

In summary, our study suggests a potential role for autophagy in controlling extracellular non-pathogenic commensal gut bacteria and may explain why ATG16L1 T300A risk allele is associated with altered gut microbial community.
Conflict of interest The authors disclose no conflict of interest.

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Compliance with ethical standards

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