Gamma-glutamyltranspeptidase expression by Helicobacter saguini, an enterohepatic Helicobacter species isolated from cotton top tamarins with chronic colitis

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Gamma-glutamyltranspeptidase expression by *Helicobacter saguini*, an enterohepatic *Helicobacter* species isolated from cotton top tamarins with chronic colitis

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Abstract

Background: *Helicobacter saguini* is a novel enterohepatic *Helicobacter* species isolated from captive cotton top tamarins with chronic colitis and colon cancer. Monoassociated *H. saguini* infection in gnotobiotic IL-10$^{-/-}$ mice causes typhlocolitis and dysplasia; however, the virulent mechanisms of this species are unknown. Gamma-glutamyltranspeptidase (GGT) is an enzymatic virulence factor expressed by pathogenic *Helicobacter* and *Campylobacter* species that inhibits host cellular proliferation and promotes inflammatory-mediated gastrointestinal pathology. The aim of this study was to determine if *H. saguini* expresses an enzymatically active GGT homologue with virulence properties.

Experimental procedures: Two putative GGT paralogs (HSGGT1 and HSGGT2) identified in the *H. saguini* genome were bioinformatically analysed to predict enzymatic functionality and virulence potential. An isogenic knockout mutant strain and purified recombinant protein of HSGGT1 were created to study enzymatic activity and virulence properties by *in vitro* biochemical and cell culture experiments.

Results: Bioinformatic analysis predicted that HSGGT1 has enzymatic functionality and is most similar to the virulent homologue expressed by *Helicobacter bilis*, whereas HSGGT2 contains putatively inactivating mutations. An isogenic knockout mutant strain and recombinant HSGGT1 protein were successfully created and demonstrated that *H. saguini* has GGT enzymatic activity. Recombinant HSGGT1 protein and sonicate from wild-type but not mutant *H. saguini* inhibited gastrointestinal epithelial and lymphocyte cell proliferation without evidence of cell death. The antiproliferative effect by *H. saguini* sonicate or recombinant HSGGT1 protein could be significantly prevented with glutamine supplementation or the GGT-selective inhibitor acivicin. Recombinant HSGGT1 protein also induced proinflammatory gene expression in colon epithelial cells.

Conclusions: This study shows that *H. saguini* may express GGT as a potential virulence factor and supports further *in vitro* and *in vitro* studies into how GGT expression by enterohepatic *Helicobacter* species influences the pathogenesis of gastrointestinal inflammatory diseases.

1 | INTRODUCTION

Enterohepatic *Helicobacter* species (EHS) are gram-negative, spiral-shaped bacteria that colonise the lower intestine, liver, and gall bladder of mammals, birds, and reptiles and are associated with the occurrence of gastrointestinal inflammatory diseases and cancers (Hansen, Thomson, Fox, el-Omar, & Hold, 2011; Mitchell et al., 2014; Thomson et al., 2011; Whary & Fox, 2004). Infection by EHS is proposed to potentiate the risk of developing inflammatory bowel disease (IBD) in animal models and humans (Hansen et al., 2011; Mitchell et al., 2014; Thomson et al., 2011; Whary & Fox, 2004), analogous to the causative relationship between *Helicobacter pylori* infection and the occurrence of gastric peptic ulcers and cancer (Marshall, 1995). *Helicobacter hepaticus* and *Helicobacter bilis* are EHS whose infection in mice are well-established models of pathogen-induced IBD and intestinal carcinogenesis (Archer et al., 1988; Fox et al., 1994; Fox et al., 1995; Fox et al., 1996a; Fox et al., 1996b; Romero et al., 1988; Ward et al., 1994). Experimental infection by *Helicobacter cinaedi* and *Helicobacter fennelliae*, both EHS originally isolated from homosexual men with proctitis, elicit diarrhoea and gastrointestinal inflammation in pigtail macaques (Flores et al., 1990; Totten et al., 1985). A current meta-analysis has also found a significant association between EHS infection and IBD status in human patients; however, specific *Helicobacter* spp. linked to human IBD remain to be elucidated (Castano-Rodriguez, Kaakoush, Lee, & Mitchell, 2015).

Our lab discovered a novel EHS, named *Helicobacter saguini*, cultured from colonic biopsy and faecal samples of captive cotton top tamarins (CTTs; *Saguinus oedipus*; Saunders et al., 1999; Shen et al., 2016). CTTs are an endangered new world primate species with a high incidence of idiopathic chronic colitis and colonic adenocarcinomas when maintained in captive colonies (Johnson, Ausman, Sehgal, & King, 1996; Saunders et al., 1999; Wood et al., 1998). The clinical and histopathological manifestations of large bowel inflammation in CTTs strongly resemble those of human ulcerative colitis, making these animals the ideal model to study etiopathogenesis of this disease. Although the protected status of CTTs has prevented establishing a direct etiological relationship between Helicobacter infection and IBD, we recently demonstrated that monoassociated infection of gnotobiotic C57BL/6 IL-10$^{-/-}$ mice with *H. saguini* induces IBD and procarcinogenic changes in the large intestine (Shen et al., 2016). These data suggest that *H. saguini* infection may influence the onset and progression of chronic colitis and colon cancer in CTTs.

Though a substantial body of evidence supports a causative association between EHS infection and IBD, the pathogenic mechanisms of these organisms remain incompletely defined. The best characterised virulence factor gene for EHS is cytolethal distending toxin (CDT), a DNA nuclease with proinflammatory and genotoxic effects to the gastrointestinal tract (Pratt, Sachen, Wood, Eaton, & Young, 2006; Shen et al., 2009; Young et al., 2004). Nevertheless, not all EHS associated with IBD encode CDT, indicating the need to identify and characterise other virulence factors expressed by these pathogens. Recently, gamma-glutamyltranspeptidase (GGT) expression by *H. pylori*, *Helicobacter suis*, *H. bilis*, and *Campylobacter jejuni* has been shown to be important for host colonisation persistence and inflammatory disease pathogenesis (Barnes et al., 2007; De Bruyne et al., 2016; Floch et al., 2014; Javed, Mejías-Luque, Kalali, Bolz, & Gerhard, 2013; Rossi et al., 2012; Wustner et al., 2017; Zhang et al., 2015). GGT is a constitutively expressed periplasmic enzyme hypothesised to promote bacterial survival by metabolising extracellular glutamine into glutamate, which is then imported by the bacterial cell to fuel energy and metabolic needs (Ling, Yeoh, & Ho, 2013; Ricci et al., 2014; Rossi et al., 2012; Shibayama et al., 2007). Our initial characterisation of *H. saguini* determined this organism does not express CDT but does display biochemical GGT activity (Shen et al., 2016). As part of our efforts to further understand the pathogenic potential of *H. saguini*, the aim of this study was to test the hypothesis that *H. saguini* expresses an enzymatically functional GGT with virulence properties.

2 | RESULTS

2.1 | *H. saguini* HSGGT1 is homologous to other *Helicobacter* and *Campylobacter* spp. GGTs with virulence properties

Our initial characterisation indicating that *H. saguini* has biochemically active GGT was substantiated by the identification of two *ggt* gene paralogs in the *H. saguini* genome, designated herein as HSGGT1 and HSGGT2. Both HSGGT1 and HSGGT2 showed considerable homology with representative bacterial and human *ggt* genes (Table 1). However, HSGGT1 had substantially more homology than HSGGT2 with the known virulence *ggt* genes from *H. bilis* (bgh2, HBGGT), *H. pylori* (HPGGT), *H. suis* (HSuGGT), and *C. jejuni* (CJGGT; Table 1). HSGGT2 was most similar to bgh1 (Table 1), a second *ggt* gene detected in *H. bilis* that contains mutations in conserved functional motifs putatively rendering it enzymatically inactive (Rossi et al., 2012). Multi-sequence alignments showed that HSGGT1 preserves all residues necessary for enzymatic function and putative virulence, including a signal sequence for periplasmic secretion, the threonine dyad for autocatalytic maturation and transpeptidase activity, and the lid loop for substrate binding (Figure 1). Conversely, HSGGT2 shared identical amino acid mutations with *H. bilis* bgh1 (Figure S1), presumably responsible for its inactivity (Rossi et al., 2012). This indicated that HSGGT1 and not HSGGT2 is most likely the enzymatically active paralog. Additionally, the predicted three-dimensional structure of HSGGT1 strongly resembled solved crystal structures of HPGGT (see Supporting Information). Together, these bioinformatics analyses suggested that HSGGT1 has enzymatic and virulence functionality. As HSGGT1 showed the greatest sequence homology to HBGGT, subsequent experiments used HBGGT as a positive control for GGT activity and virulence.

2.2 | Construction of isogenic *H. saguini* GGT-knockout mutant (HSΔGGT1)

To test the virulence potential of HSGGT1, an isogenic GGT-knockout mutant of *H. saguini* (HSΔGGT1) was created for comparison against the wild-type strain. The HSGGT1 gene was successfully

TABLE 1 Amino acid % sequence homology of bacterial and human *ggt* genes

	H. saguini HSGGT1	*H. saguini* HSGGT2	*H. bilis* bgh2, HBGGT	*H. bilis* bgh1	*C. jejuni* CJGGT	*H. suis* HSuGGT	*H. pylori* HPGGT	*E. coli* ECGGT	*Homo Sapiens* hGGT
H. saguini HSGGT1	100.00	77.22	86.93	79.85	82.73	81.31	80.62	67.35	48.04
H. saguini HSGGT2	—	100.00	73.54	79.68	74.69	71.91	72.34	66.73	50.28
H. bilis bgh2, HBGGT	—	—	100.00	79.96	82.97	81.38	78.61	63.79	46.15
H. bilis bgh1	—	—	—	100.00	77.54	76.37	75.40	64.99	47.32
C. jejuni CJGGT	—	—	—	—	100.00	82.67	81.15	67.90	51.77
H. suis HSuGGT	—	—	—	—	—	100.00	86.02	66.78	49.81
H. pylori HPGGT	—	—	—	—	—	—	100.00	66.49	50.29
E. coli ECGGT	—	—	—	—	—	—	—	100.00	52.53
Homo sapiens hGGT	—	—	—	—	—	—	—	—	100.00

H. saguini HSGGT1 and HSGGT2, *H. bilis* ATCC 43879 bgh1 (GenBank: EEO24284.1) and bgh2 (GenBank: EEO24771.2), *H. pylori* 26695 (NCBI Reference Sequence: NP_207909.1), *H. suis* (GenBank: ADF28653.1), *C. jejuni* strain 81116 (GenBank: AAV30679.1), *E. coli* str. K-12 (NCBI Reference Sequence: NP_417904.1), and human (NCBI Reference Sequence: NP_005256.2) GGT amino acid homology comparison with Protein Blast (Altschul, et al. 1997).

FIGURE 1 Multisequence alignment of bacterial and human *ggt* genes generated with Clustal Omega (Sievers et al., 2011). Signal sequence predicted with SignalP 4.1 Server using the "Sensitive (reproduce SignalP 3.0's sensitivity)" setting (Petersen, Brunak, von Heijne, & Nielsen, 2011). Green highlighted residues designate the signal sequence exclusion site. Yellow highlighted residues and brackets designate disulfide bonds. Orange highlighted residues designate the autocatalytic cleavage site. Grey highlighted residues designate the conserved GXXGGXXI motif. Purple-coloured font residues designate the threonine catalytic dyad. Red-coloured font residues designate the lid loop. Boxed residues designate the substrate binding and processing sites. Asterisks (*) underneath the sequences indicate positions in which amino acids are conserved between all aligned sequences. Colons (:) underneath the sequence indicate positions in which there are conserved amino acid substitutions between aligned sequences. Dots (.) underneath the sequences indicate positions in which there are semiconserved amino acid substitutions between aligned sequences

replaced with *catNT*, a chloramphenicol resistance gene (Figure S3). Flanking upstream and downstream gene expression at the site of mutagenesis was detected in the wild-type and mutant strains, indicating a polar effect was not induced (Figure S3E). Likewise, HSGGT1 gene expression was detected in the wild-type, but not the mutant strain (Figure S3E). Mutant bacteria grew normally *in vitro*, consistent with other GGT-knockout *Helicobacter* spp. mutants (Chevalier, Thiberge, Ferrero, & Labigne, 1999; Rossi et al., 2012). *H. bilis* (HB) and wild-type *H. saguini* (HS) sonicate cleaved the glutamate substrate analogue GpNA into pNA at comparable levels (Figure 2a) that was completely abolished for both by the GGT-specific inhibitor acivicin (Figure 2b) or boiling for 30 min (data not shown). Conversely, GGT activity was undetectable in sonicated HSΔGGT1 (Figure 2b). Live *H. saguini* wild-type but not HSΔGGT1 metabolised GpNA (Figure S4A) as well as produced glutamate in DMEM media (Figure S4B). These findings using live and sonicated HSΔGGT1 also indicate that HSGGT2 does not have detectable GGT enzymatic activity. Interestingly, GGT activity was magnitudes less in live *H. saguini* and *H. bilis* compared with their sonicate preparations. This could represent that GGT enzyme activity or protein concentrations are higher in sonicate preparation compared with live bacteria under *in vitro* conditions.

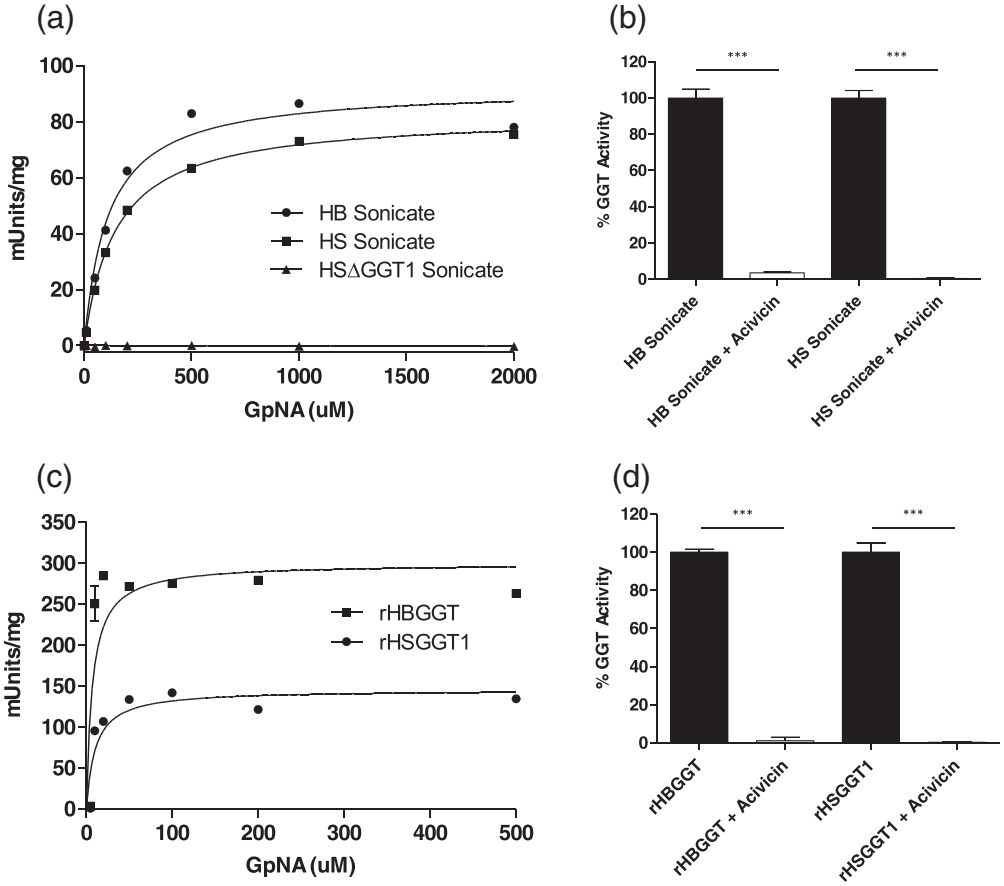

FIGURE 2 (a) HB and HS sonicates exhibited comparable levels of GGT activity. No GGT activity was detected by HSΔGGT1 sonicate, indicating successful removal of the functional *ggt* gene. Results shown for representative curve performed in duplicate at each GpNA concentration. (b) GGT activity by HB and HS sonicates at 2000 μM of GpNA was completely inhibited by pretreatment with the GGT-specific inhibitor acivicin (pretreatment, 30-min incubation with 0- or 1-mM acivicin at 37°C). Results shown for two experiments performed in duplicate. (c) Partially purified rHBGGT and rHSGGT1 proteins from fraction #3 exhibited GGT activity. Enzyme activity for both rGGTs conformed to the Michaelis–Menten kinetic profile. Binding affinities to the substrate GpNA for rHBGGT and rHSGGT1 were 6.73 ± 0.31 and 12.3 ± 2.35 μM, respectively. Likely due to a higher concentration of mature enzyme (see Figure S5), rHBGGT had a higher transpeptidase V_{max} of 299.6 mUnits/mg compared with 145.6 mUnits/mg for rHSGGT1. Results shown for representative curve performed in duplicate at each GpNA concentration. (d) GGT activity by rHBGGT and rHSGGT1 at 2,000 μM of GpNA was completely inhibited by pretreatment with the GGT-specific inhibitor acivicin (pretreatment, 30 min incubation with 0- or 1-mM acivicin at 37°C). Results shown for two experiments performed in duplicate

2.3 | Purification of enzymatically functional rHBGGT and rHSGGT1 proteins

Purified recombinant His-tagged *H. bilis* GGT (rHBGGT) and *H. saguini* GGT (rHSGGT1) proteins yielded prominent protein bands at ~60, ~40, and ~20 kDa (Figure S5A,B), consistent with other purified *Helicobacter* and *Campylobacter* spp. GGT (Flahou et al., 2011; Floch et al., 2014; Rossi et al., 2012). Whereas rHBGGT appeared maturated immediately after purification, rHSGGT1 matured into detectable 40- and 20-kDa subunits by 24-hr incubation at 37°C (Figure S5C,D).

rHBGGT and rHSGGT1 exhibited GGT activity (Figure 2c) that was completely ablated by pretreatment with acivicin (Figure 2d) or boiling (data not shown). The mean binding affinity (Km) of rHBGGT for GpNA from two separate batches was 6.73 ± 0.31 μM, consistent to that of 7.7 ± 1.2 μM as previously reported (Bolz et al., 2016; Rossi et al., 2012). Three separate batches of rHSGGT1 had a mean Km of 12.3 ± 2.35 μM, which is of similar magnitude to both rHPGGT (reported as 9.8 ± 1.5 μM; Bolz et al., 2016; Rossi et al., 2012) and rHBGGT. rHBGGT had about twofold higher transpeptidase activity compared with rHSGGT1 (Vmax of 299.6-mUnit/mg protein for rHBGGT versus 145.6-mUnit/mg protein for rHSGGT1), which agrees with a previous report showing the magnitude of GGT activity is dependent on the degree of enzyme maturation (Boanca, Sand, & Barycki, 2006).

2.4 | HSGGT1 inhibits intestinal epithelial and lymphocyte cellular proliferation

As both *H. bilis* and *H. saguini* colonise, the mucosal epithelium of the large intestine, the effects of HBGGT and HSGGT1 on colon epithelial cells were tested. HB and HS sonicates and rGGT proteins significantly blocked proliferation of HT-29 cells (Figure 3a,b). Wild-type HS sonicate also caused statistically greater inhibition of proliferation compared with HSΔGGT1 sonicate treatment by 48 hr (Figure 3a).

Interestingly, HSΔGGT1 sonicate still yielded significant antiproliferative effects compared with the PBS control, suggesting that *H. saguini* may express other antiproliferative factors besides HSGGT1 (Figure 3a). Though not readily apparent from the genomic or biochemical characterisation, these other factors will require further investigation to identify. HB and HS sonicates and rGGT proteins also blocked proliferation in a dose-dependent manner by 72 hr of treatment (Figure 3c,d). Interestingly, live infection by *H. saguini* for 72 hr did not affect HT-29 cell proliferation (data not shown). HB and HS sonicates and rGGT treatment also blocked proliferation of AGS gastric epithelial cells (Figure S6), HeLa cervical epithelial cells (Figure S7), and T84 colonic epithelial cells (Figure S8) in a time- and dose-dependent manner.

Helicobacter spp. and *C. jejuni* GGTs have been shown to impair the proliferation of lymphocytes as well. HS sonicate and rGGT proteins significantly blocked Jurkat T cell proliferation by 48 hr (Figure 4a,b). Furthermore, the antiproliferative effects caused by wild-type HS sonicate treatment were statistically greater versus HSΔGGT1 sonicate at 48 and 72 hr (Figure 4a). Whereas rHBGGT blocked T cell proliferation (Figure 4B), HB sonicate displayed significant cytotoxicity (Figure 4a). This effect may be due to expression of CDT, which is expressed by *H. bilis* (Chien et al., 2000; Kostia et al., 2003) and has been shown to induce apoptosis in lymphocytes and other cell types (Pratt et al., 2006; Shen et al., 2009). T cells, therefore, appear to be more sensitive to cytotoxicity by HB sonicate than the antiproliferative effects of HBGGT. The effects of HB and HS sonicates and rGGT protein treatments on T cells were dose dependent (Figure 4c,d).

Contrary to reports that have shown that HPGGT and HSuGGT can induce apoptosis in epithelial cells, observable indicators of cytotoxicity such as megalocytosis (abnormal cell body enlargement), cellular rounding, membrane blebbing, and plate detachment were not evident in the epithelial cell lines tested after 72 hr of treatment with sonicate and rGGT protein (data not shown). Likewise, cell viability, as measured by trypan blue exclusion, was unaffected by sonicate or rGGT protein treatment (data not shown). Therefore, HBGGT and HSGGT1 appear to inhibit epithelial proliferation without causing overt cell death. This is in agreement with previous reports showing that HBGGT and CJGGT did not induce cell death in intestinal epithelial cells and that cell death caused by HPGGT may be an artefact due to serum starvation (Floch et al., 2014; Rossi et al., 2012).

2.5 | Glutamine supplementation and GGT enzyme inhibition prevent antiproliferative effect

Glutamine is an essential nutrient for intestinal epithelial cell proliferation because it serves as an energy source and precursor for *de novo* nucleic acid and amino acid biosynthesis (DeBerardinis & Cheng, 2010; Kim & Kim, 2017). Restriction of glutamine has been shown to impair the proliferation of HT-29 and other intestinal epithelial cell lines *in vitro* (Rhoads et al., 1997; Wiren, Magnusson, & Larsson, 1998). Therefore, we hypothesised that the antiproliferative effect caused by *H. saguini* is due to GGT-mediated metabolism of glutamine into glutamate and that glutamine supplementation and/or inhibition of GGT enzyme activity could restore proliferation.

To exclude that glutamate produced by HSGGT1 activity (Figure S4B) affected cell proliferation, we found that incubating HT-29 cells with up to 10 mM of glutamate for 72 hr had no effect on proliferation (data not shown). Media supplemented with glutamine partially but significantly restored proliferation after sonicate or rGGTs treatment; however, proliferation was still significantly less compared with

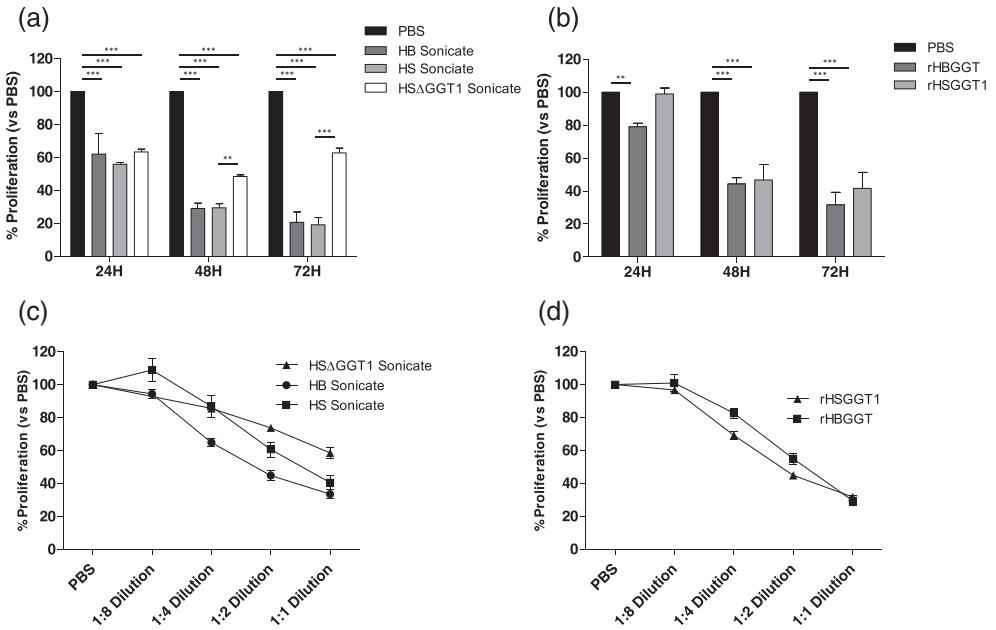

FIGURE 3 Cellular proliferation of HT-29 cells (a,b) after treatment with 0.5 mUnits of Helicobacter sonicate or rGGT for 24, 48, and 72 hr. Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Cellular proliferation of HT-29 cells (c,d) after treatment with increasing doses of Helicobacter sonicate or rGGT for 72 hr. Results shown for three experiments performed in triplicate

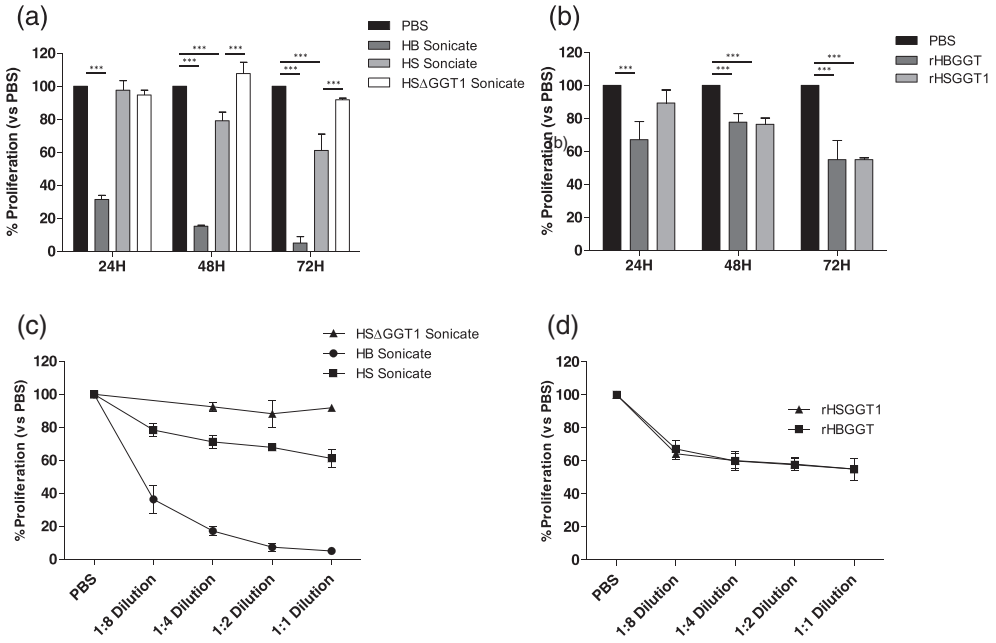

FIGURE 4 Cellular proliferation of Jurkat T cells (a,b) after treatment with 0.5 mUnits of Helicobacter sonicate or rGGT for 24, 48, and 72 hr. Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Cellular proliferation of Jurkat T cells (c,d) after treatment with increasing doses of Helicobacter sonicate or rGGT for 72 hr. Results shown for three experiments performed in triplicate

controls cells (Figure 5a). To test if the antiproliferative effect of HSGGT1 was dependent on enzyme activity, HT-29 cells were treated with sonicate and rGGT proteins pretreated with acivicin. As inferred by glutamate production in media, acivicin significantly reduced GGT activity during sonicate and rGGT protein treatment (Figure 5b). GGT inhibition also significantly restored cell proliferation (Figure 5c). Boiling sonicate and rGGT proteins rescued cell proliferation as well (Figure S9). Together, these results suggest that the antiproliferative effect of HSGGT1 and HBGGT is in part due to the enzymatic metabolism of glutamine.

2.6 | rHSGGT1 induces proinflammatory gene expression

GGTs from *Helicobacter* spp. are capable of inducing proinflammatory changes *in vitro* and *in vivo*. HPGGT and HBGGT both increase activation of NF-κB and IL-8 expression (Gong, Ling, Lui, Yeoh, & Ho, 2010; Javed et al., 2013), whereas infection by GGT-knockout *H. pylori* and *H. suis* mutants causes significantly less inflammatory-mediated pathology in the rodent stomach compared with wild-type strains (Zhang, Ducatelle, et al., 2015). Therefore, we assessed the proinflammatory potential of HSGGT1 towards HT-29 cells. Interestingly, there was no statistical difference in IL-8 or TNF-α gene expression levels between infections with *H. saguini* wild-type versus HSΔGGT1, even though both cause significantly higher expression compared with the media control (Figure 6a). However, treatment with rHSGGT1 or rHBGGT significantly increased expression of IL-8 and TNF-α (Figure 6b).

Nevertheless, although these data show that HSGGT1 is capable of promoting proinflammatory gene expression in colon epithelial cells, it suggests that factors other than HSGGT1 may be primarily responsible for inflammatory processes in vitro.

3 | DISCUSSION

Recently, we demonstrated that *H. saguini* infection in a gnotobiotic IL-10$^{-/-}$ mouse model elicits inflammatory and dysplastic changes to the large intestine, suggesting an etiological role of *H. saguini* infection in the pathogenesis of IBD in captive CTTs (Shen et al., 2016). Although EHS infection is strongly associated with gastrointestinal inflammation and cancer progression, the mechanisms by which *H. saguini* and other EHS colonise their host and cause disease are only elucidated in part and require further studies (Shen et al., 2009; Whary & Fox, 2004; Young et al., 2004). CDT is the best characterised virulence factor for EHS, but not all EHS associated with IBD including *H. saguini* harbour this cytotoxin. According to our genomic analysis though, *H. saguini* contains homologues of other known *Helicobacter* and *Campylobacter* spp. virulence genes including flagella components, flavodoxin (*fldA*), the secreted serine protease *htrA*, type VI secretion components, arginase, and *ggt* (Shen et al., 2016). Of these, GGT appears to be important because its activity has been shown to promote hose colonisation, proinflammatory responses, and intestinal pathology. Furthermore, biochemical characterisation of *H. saguini* detected GGT activity (Shen et al., 2016). Therefore, the aim of this study was to determine if *H. saguini* expresses an enzymatically active GGT gene with virulence properties.

Two *ggt* paralogs were identified in the genome of *H. saguini*. The HSGGT1 gene, but not the HSGGT2 paralog, showed substantial homology and conservation of all residues necessary for enzymatic

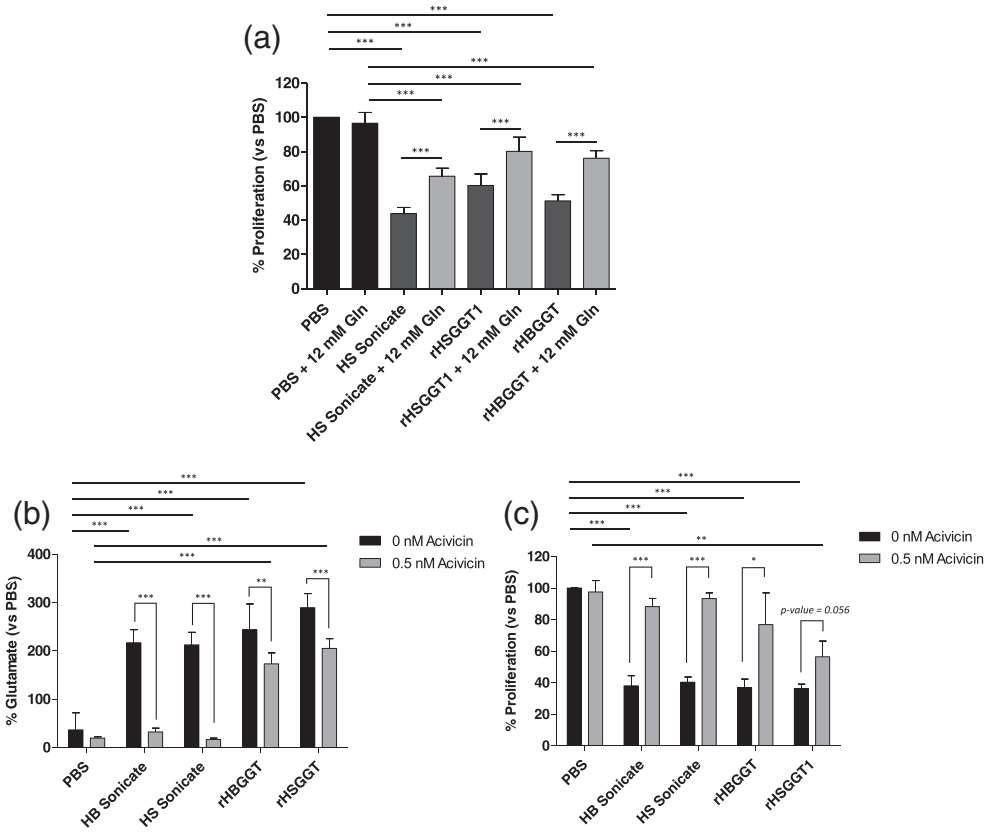

FIGURE 5 (a) Cellular proliferation of HT-29 cells after treatment with 0.5 mUnits Helicobacter sonicate or rGGT in media supplemented with 12 mM of glutamine (Gln) for 72 hr. Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Results shown for four experiments performed in triplicate. (b) Glutamate levels in DMEM media after 72-hr incubation with 0.5 mUnits Helicobacter sonicate or rGGT that pretreated with acivicin (pretreatment, 24-hr incubation with 0.0- or 0.5-nM acivicin at 37°C). Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Results of one representative experiment are shown. (c) Cellular proliferation of HT-29 cells after 72-hr treatment with 0.5 mUnits Helicobacter sonicate or rGGT that pretreated with acivicin (pretreatment, 24-hr incubation with 0.0- or 0.5-nM acivicin at 37°C). Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Results shown for three experiments performed in triplicate

function and putative virulence. Thus, we hypothesised that HSGGT1 has enzymatic and virulent properties. The finding of two *ggt* genes for *H. saguini* agrees with a previous report that *H. bilis* also contains two *ggt* genes paralog, but only one of which (bhg2, HBGGT) was found to have enzymatic and virulence activity (Rossi et al., 2012). The role of these additional *ggt* genes in *H. saguini* and *H. bilis* requires further study (Rossi et al., 2012).

To test our hypothesis, we created a viable isogenic knockout of *H. saguini* that lacked GGT activity. This finding also supports our bioinformatic predictions that HSGGT2 is enzymatically nonfunctional because the mutant *H. saguini* strain still harbours this gene, but lacked GGT activity. Likewise, we purified enzymatically active rHSGGT1 protein that metabolised the GGT substrate analogue GpNA and was blocked by the GGT-selective inhibitor acivicin. Purified rHSGGT1 had a similar binding affinity for GpNA as rHBGGT and rHPGGT but appeared to autocatalytically mature into ~40- and ~20-kDa subunits less efficiently than rHBGGT. The purification protocol in this study was modelled after that used to purify rHBGGT (Rossi et al., 2012) and as a consequence may have not been optimised for complete rHSGGT1 maturation. Nevertheless, rHSGGT1 was able to undergo the expected maturation after 24-hr incubation at 37°C.

The common *in vitro* effect shared by virulent GGTs is the ability to impair gastrointestinal epithelial and lymphocyte proliferation. We found that HS sonicate and rHSGGT1 significantly inhibited HT-29, T84, AGS, HeLa, and Jurkat T cell proliferation on par with HBGGT. Like HBGGT and CJGGT, the antiproliferative effect by HSGGT1 occurred without evidence of cell death (Floch et al., 2014; Rossi et al., 2012). We also showed that rHSGGT1 alone is capable of stimulating significant chemokine and cytokine gene expression by colon epithelial cells, consistent with the proinflammatory nature of HPGGT, HSuGGT, and HBGGT. Together, these data show that HSGGT1 exhibits enzymatic GGT activity with potential virulence properties *in vitro*.

Unexpectedly, we were unable to detect an antiproliferative or proinflammatory response by live wild-type *H. saguini* compared with the isogenic knockout, suggesting that enzymatic activity or the concentration of HSGGT1 in sonicate or rGGT preparations is not representative of live bacteria. However, it is appreciated that although some virulence factors like CDT can only be detected *in vitro* using sonicate preparation and not live bacteria, these factors have influential effects on colonisation dynamics, inflammatory pathology, and carcinogenesis using *in vivo* animal models (Fox et al., 2004; Ge et al., 2018; Shen et al., 2009; Young et al., 2004). Further studies are needed to validate the role of HSGGT1 as a virulence factor.

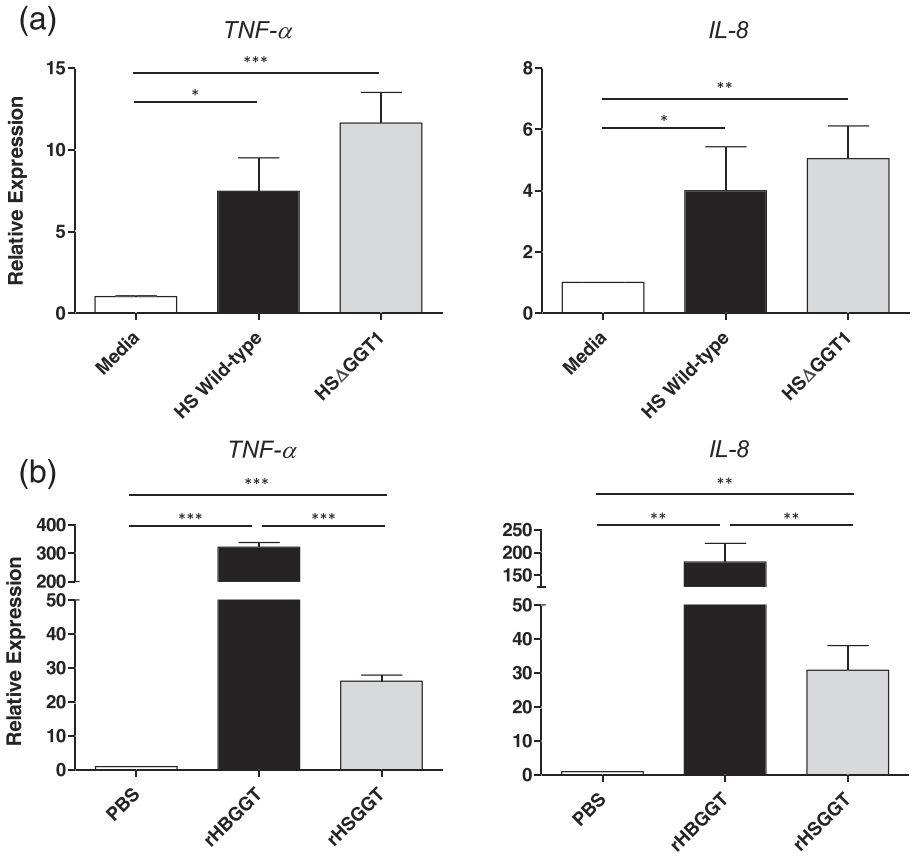

FIGURE 6 (a) TNF-α and IL-8 gene expression by HT-29 cells after 4-hr infection with live *H. saguini* wild-type or HSΔGGT1 at MOI 100. Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Results shown for three experiments performed in triplicate. (b) TNF-α and IL-8 gene expression by HT-29 cells after 4-hr treatment with 5.0 mUnits of rHBGGT and rHSGGT1. Asterisk (*) designates statistical difference (*, *P* value < 0.05; **, *P* value < 0.01; ***, *P* value < 0.001) between indicated groups. Results shown for three experiments performed in triplicate

GGT expression has been shown to be pivotal for infection and disease pathogenesis by *H. pylori*, *H. suis*, and *C. jejuni* (Chevalier et al., 1999; Rossi et al., 2012; Zhang et al., 2013). GGT-knockout mutants of *H. pylori* and *C. jejuni* have an impaired ability to establish colonisation and cause pathology in their hosts (Barnes et al., 2007; Chevalier et al., 1999; Wustner et al., 2017; Zhang et al., 2013). In contrast, GGT expression by *H. suis* had no effect on stomach colonisation levels in mice or Mongolian gerbils but causes more severe inflammation and pathology compared with the GGT-knockout mutant (Zhang et al., 2013). In human patients, the occurrence of peptic ulcer disease is also associated with higher GGT activity by *H. pylori* (Park et al., 2014). Whether GGT endows *H. bilis* with similar pathogenic properties *in vivo* has not been published to date.

The putative virulence mechanism of GGT is proposed to be due to enzymatic degradation of host glutamine into glutamate (Schmees et al., 2007; Shibayama et al., 2007; Wustner et al., 2015). Glutamate is utilised by the bacteria to fuel metabolism, whereas intestinal cells become deprived of glutamine, a critical precursor for nucleotide and amino acid biosynthesis that is required for proliferation and other cellular functions. Dietary glutamine supplementation has been found to attenuate gastric inflammation and pathology in mice and Mongolian gerbils infected with *H. pylori* or *H. suis*, reinforcing the hypothesis that GGT acts by depleting host glutamine availability (De Bruyne et al., 2016; Hagen et al., 2009; Wustner et al., 2017). Additionally, dietary glutamine supplementation on its own has been shown to significantly augment colonic epithelial cell proliferation *in vivo* in rats (Mandir & Goodlad, 1999).

Our *in vitro* findings suggest the antiproliferative effects of HSGGT1 to colon epithelial cells could contribute to the deterioration of intestinal barrier integrity. Impaired barrier function is a defining characteristic of IBD, and it is hypothesised to initiate and promote unregulated and sustained proinflammatory responses by allowing excessive penetration of luminal bacteria and foreign antigens into the lamina propria (Abraham & Cho, 2009; Martini, Krug, Siegmund, Neurath, & Becker, 2017; Thoreson & Cullen, 2007; Xavier & Podolsky, 2007). Likewise, although *H. saguini* and other *Helicobacter* spp. directly colonise the mucosal and epithelial surface of the gastrointestinal tract *in vivo*, our *in vitro* data suggest that GGT activity could also impair lymphocyte proliferation. As described for other *Helicobacter* spp. GGTs, targeting lymphocytes may suppress the host's immune response and thereby facilitate colonisation in the intestine. Declining barrier integrity may allow *Helicobacter* spp. to invade intestinal lymphoid tissue where GGT can directly target lymphocytes. Alternatively, GGT from *H. suis* has been shown to be transported by outer membrane vesicles into the lamina propria to impact immune cells (Zhang et al., 2013).

In conclusion, we have shown *H. saguini* expresses an enzymatically active GGT homologue with potential virulence properties.

Although GGT genes are annotated in numerous other enterohepatic *Helicobacter* spp. genomes, the virulence properties of this gene have only been experimentally validated for two EHS, *H. bilis* and now *H. saguini*. The multimodal influences of GGT, including colonisation persistence and inflammation, suggest that it could be important in *Helicobacter* spp.-induced IBD pathogenesis. Aside from EHS, other *Proteobacteria* species in the gut microbiome may also express GGTs as virulence factors that influence IBD. Future studies in our lab aim to evaluate the role of HSGGT1 *in vivo* using our gnotobiotic mouse model of IBD. Most importantly, GGT may represent a novel pharmacological target for antibiotics and vaccines (Bolz et al., 2016; Zhang et al., 2015) to treat and prevent *Helicobacter* spp. and *Campylobacter* spp. infection in veterinary and human patients.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial and cell culture

H. saguini MIT 97-6194-5 and *H. bilis* strain ATCC 43879 were cultured on trypticase soy agar plates with 5% sheep blood agar plates in microaerobic conditions (10% CO_2, 10% H_2, 80% N_2) at 37°C and collected after 48 hr, which corresponds to the mid-exponential phase of bacteria growth. Contaminating colonies in aerobic and anaerobic culture plates or by Gram staining were not detected, ensuring the purity of the bacteria preparations. HeLa S3 cervical epithelial cells (ATCC CCL2.2), AGS stomach cancer epithelial cells (ATCC CRL-1739), HT-29 colon cancer epithelial cells (ATCC HTB-38), T84 colon cancer epithelial cells (ATCC CCL-248), and Jurkat T cells (ATCC TIB-152) were grown and maintained in Eagle's Minimum Essential Medium (ATCC, Manassas, VA), Dulbecco's Modified Eagle's Medium (ATCC), or RPMI-1640 Medium (ATCC) containing 10% Fetal Calf Serum (Sigma-Aldrich, St. Louis, MO) and 1% Antibiotic-Antimycotic (100-unit/ml penicillin, 100-μg/ml streptomycin, and 0.25-μg/ml amphotericin B; Gibco/Thermo Fisher Scientific, Grand Island, NY) at 37°C with 5% CO_2.

4.2 | Construction of an isogenic GGT-knockout mutant (HSΔGGT1)

A full description of this method is described in the Supporting Information. Briefly, 500-bp fragments upstream and downstream of the *H. saguini* HSGGT1 gene were amplified and spliced together with an intervening HincII restriction enzyme site by overlap extension PCR (Heckman & Pease, 2007; Lee, Shin, Ryu, Kim, & Ryu, 2010). After inserting this product into a pCR2.1-TOPO vector, a chloramphenicol resistance gene cassette (*catNT*) was inserted utilising the HincII restriction enzyme site. The recombinant plasmid was transformed into *H. saguini* by electroporation. Mutants were selected as previously described on blood agar plates containing 25-μg/ml chloramphenicol under microaerobic conditions (Ge et al., 2005; Ge et al., 2008; Ge et al., 2014). Mutants were confirmed for genetic authenticity by PCR amplification and sequencing. To rule out a polar effect on upstream and downstream gene expression at the mutagenesis site, cDNA from these genes was amplified from the wild-type and mutant strains.

4.3 | Preparation of crude bacterial sonicate

After reaching the mid-exponential phase of growth (about 48 hr), bacteria were collected from sheep blood agar plates into sterile PBS and washed once with PBS by centrifuging samples at 13,523 × *g* for 5 min at room temperature. Pellets were resuspended in 2 ml of PBS and then sonicated on ice using the following programme: amplitude, 35; power, 7 W; 30-s intervals for a total of 5 min with 1-min breaks between intervals (QSonica Sonicator, Newton, CT). Sonicate samples were centrifuged at 13,523 × *g* for 10 min at 4°C to pellet large debris. Supernatant was collected and then filter-sterilised through 0.2-μm filters. Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Crude sonicate samples were stored at −80°C until use.

4.4 | Expression and purification of N-terminal His-tagged recombinant HSGGT1 and HBGGT

A full description of this method section is described in the Supporting Information. Briefly, HSGGT1 and HBGGT genes, without the signal sequence, were amplified by PCR from genomic DNA of *H. saguini* and *H. bilis* strain ATCC 43879, respectively to contain complementary overlapping sequences to the pET-46 expression vector. Genes were cloned into pET-46 expression vectors and transformed into BL21(DE3) competent *Escherichia coli* cells as the expression hosts, following the manufacture's Ek/LIC Vector Kit protocol (Novagen. Madison, WI).

Expression of the recombinant HSGGT1 and HBGGT proteins (rHSGGT1 and rHBGGT) with N-terminal 6×-His tags was induced by treatment with 0.4-mM IPTG for 4 hr at 37°C. Bacteria were pelleted at 10,800 × *g* for 10 min at 4°C and washed once with PBS. Pellets were resuspended in ice-cold binding buffer (50-mM Tris–HCl, 500-mM NaCl, 20-mM imidazole, pH 7.5) containing one 100× Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and sonicated as described above. Supernatant was then filter sterilised through 0.2-μm filters and loaded on HisTrap column (GE Healthcare Biosciences, Westborough, MA) at 1 ml/min at 4°C. Columns were washed with binding buffer prior to eluting bound proteins with an imidazole gradient of 50, 100, 250, and 500 mM in elution buffer (50-mM Tris–HCl, 500-mM NaCl, pH 7.5). Eluted fractions were collected and tested for purity by SDS-PAGE. Fractions containing the expected GGT subunit proteins were buffer exchanged and concentrated into PBS using 3-kDa Amicon Ultra 2-ml Centrifugal Filters (EMD Millipore, Billerica, MA). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. To track autocatalytic maturation of GGT, 15-μg protein aliquots were incubated at 37°C between 0 and 24 hr. At the indicated time points, aliquots were immediately boiled in reducing buffer for 5 min and then run on an SDS-PAGE to visualise degree of maturation. Fractions were tested for GGT enzymatic activity as described below. Fractions were then aliquoted and stored at −80°C until used.

4.5 | Enzyme assay for GGT activity

GGT transpeptidase enzymatic activity of sonicated *Helicobacter* spp. samples and recombinant GGT proteins was determined by measuring the cleavage of L-γ-glutamyl-p nitroanilide (GpNA; Sigma-Aldrich) into 4-p-nitroaniline (pNA). Two hundred-microlitre reactions containing 5, 1, or 0.5 μg of protein, 20 mM of glycylglycine (Sigma-Aldrich), and 0–2,000 μM of GpNA in Tris–HCl buffer (pH 8) were incubated at 37°C for 30 min. Recombinant proteins were preincubated for 24 hr at 37°C prior to performing the assay to increase enzyme maturation. Reactions were performed in duplicate. Enzymatic cleavage into 4-p-nitroaniline (pNA) was quantified by reading optical density at 405 nm for 30 min in 30-s intervals with a SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Optical densities were corrected against PBS controls to account for spontaneous autolysis of GpNA into pNA. Replicates were averaged together, and concentrations of pNA were calculated according to Beer–Lambert Law using the reported extinction coefficient of 8,800 M^{-1} cm^{-1}. Enzyme activity was calculated as mUnits/mg protein, in which one unit was defined as the quantity of enzyme that catalyses the formation of one μmole of pNA per minute. Enzyme kinetics (K_M and V_{max}) were determined by fitting the data to the Michaelis–Menten equation using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). The transpeptidase reactions were repeated using samples preincubated with acivicin (Cayman Chemical, Ann Arbor, MI) for 30 min at 37°C prior to addition to substrates. GGT activity was also tested in 5×10^5–4×10^6 live Helicobacter cells (estimated according optical density at 600 nm) collected after 48 hr of growth, as described above, and reported as mUnits/10^6 cells.

4.6 | MTT assay for quantification of cell proliferation

Five thousand HeLa, HT-29, or AGS, or 10,000 T84 or T cells were plated in 100 μl of media in 96-well and incubated for 24 hr at 37°C with 5% CO_2. Cells were treated with 0.5, 0.25, 0.125, 0.0625, and 0 (i.e., PBS control) mUnits doses of crude *H. saguini* wild-type (HS), HSΔGGT1, and *H. bilis* (HB) sonicates and rHSGGT1 and rHBGGT protein in triplicate for 24, 48, and 72 hr. Following incubation, cell proliferation was assessed using the MTT assay (Vybrant/Thermo Fisher Scientific). Media was aspirated from the wells and replaced with 12 mM of MTT reagent dissolved in non-phenol red MEM media (Gibco) containing 10% Fetal Calf Serum (Sigma-Aldrich). Cells were incubated at 37°C with 5% CO_2 for 4 hr. Following incubation with the MTT regent, media was aspirated and replaced with 20-mM HCl and 0.1% Nondent P-40 (NP-40) in isopropanol. The solution was gently pipetted to dissolve formazan crystal. Plates were incubated at 37°C with 5% CO_2 for 10 min. Optical density was read at 540 nm. Optical densities were blank corrected, and replicates were averaged together. Viability was expressed as a percentage compared with the PBS control. In parallel experiments, plates were stained with Diffquick stain (Thermo Scientific) after 72 hr of incubation for microscopic assessment of cell confluence and morphology using a Zeiss Axiovert-10 microscope (Zeiss, Germany).

4.7 | Trypan blue exclusion assay for cell viability

Cell viability was measured using trypan blue, a dye that is excluded by viable cells due to intact membrane integrity (Strober, n.d.; Strober, 2001). Fifty thousand HT-29 cells were plated in 1 ml of media in 24-well plates for 24 hr and then treated with 5 mUnits of HS and HSΔGGT1 sonicates or rHSGGT1 and rHBGGT protein for 72 hr. Cells were treated with 0.1% saponin (Sigma-Aldrich) for 5 min as a positive control for cell death. Media supernatant was collected and pelleted at 13,523 × *g* for 5 min to collect any floating or dead cells. Cells were detached from the plate with 0.25% trypsin–EDTA (Gibco) for 5 min and recombined with their supernatant pellets. Five microlitres of cells were mixed with 5 μl of 0.4% trypan blue (Gibco) and then loaded into a haemocytometer to count live and dead cells under a light microscope.

4.8 | Proinflammatory cytokine expression

Fifty thousand HT-29 cells were plated in 1 ml of media in 24-well plates. Cells were incubated for 24 hr at 37°C with 5% CO_2 to allow cells to adhere to the plates. Cells were treated with 5.0 mUnits dose of rHSGGT1, rHBGGT, or PBS for 4 hr at 37°C with 5% CO_2 in duplicate. Cells were collected in 1 ml of Trizol reagent (Invitrogen/Thermo Fisher Scientific) for total RNA extraction following the manufacturer's protocol. RNA was extracted using Trizol reagent. Total RNA (5 μg) was converted into cDNA using a high capacity cDNA Archive kit following the manufacturer's protocol (Applied Biosystems, Foster City, CA). cDNA levels for TNF-α (Probe ID: HS99999043_M1; Thermo Fisher Scientific) and IL-8 (Probe ID: HS00174103_M1; Thermo Fisher Scientific) mRNA were measured by quantitative PCR using commercial primers and probes for each cytokine. Briefly, duplicate 20-μl reactions contained 4 μl of cDNA, 1 μl of a commercial 20× primer-probe solution, 10 μl of 2× master mix (Applied Biosystems), and 5 μl of double-distilled H_2O. Relative expression of mRNA was calculated using the comparative C_T method with RNA input standardised between samples by expression levels of the endogenous reference gene, GAPDH (Probe ID: Hs99999905_M1; Thermo Fisher Scientific). Mean fold changes from three separate experiments were plotted between treated and PBS control cells.

4.9 | *In vitro* infection by *H. saguini* on HT-29 proliferation and proinflammatory cytokine expression

For *in vitro* infection experiments, *H. saguini* wild-type or ΔGGT1 were collected in 1% FBS DMEM media without antibiotic after 48 hr of growth and adjusted to an optical density at 600 nm corresponding to a multiplicity of infection (the number of bacteria per cell at the onset of infection) of 100. Five thousand HT-29 cells grown in 96-well plates and 10^6 HT-29 cells grown in six-well plates for 24 hr were used for proliferation and proinflammatory cytokine expression experiments, respectively. The plates were centrifuged at 200 × *g* to facilitate bacterial cell adhesion and then incubated at 37°Cunder 5% CO_2. Cell proliferation was quantified after 72 hr using the MTT assay as described above. Proinflammatory cytokine expression was

assessed after 4 hr of infection. Media supernatant was collected for quantification of glutamate levels using the colorimetric Glutamate Assay Kit (Cell Biolabs, Inc., San Diego, CA) following the manufacture's instruction. HT-29 cells were washed once with phosphate-buffered saline (PBS) and collected in TRIzol reagent for total RNA extraction and qPCR of TNF-α and IL-8, as described above.

4.10 | Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance with a Tukey post-hoc test using GraphPad Prism 5.0 (GraphPad Software, Inc.). Results were considered significant at P value < 0.05. All graphs were generated using GraphPad Prism 5.0 (GraphPad Software, Inc.).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION

A. M. designed and performed the experiments, analysed and interpreted the data, and wrote the manuscript. Z. S., Y. F., and Z. G. assisted with experimental design and construction of *H. saguini* mutant strain. S. C. A. generated and analysed the three-dimensional structures of HPGGT and predicted HSGGT1. K. R. assisted with the experimental design and purification of rGGT proteins. J. F. G. supervised the projects, interpreted the data, and reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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