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# Gastric colonisation with a restricted commensal microbiota replicates the promotion of neoplastic lesions by diverse intestinal microbiota in the *Helicobacter pylori* INS-GAS mouse model of gastric carcinogenesis

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## Abstract

**Objectives—**Gastric colonisation with intestinal flora (IF) has been shown to promote *Helicobacter pylori* (*Hp*)-associated gastric cancer. However, it is unknown if the mechanism involves colonisation with specific or diverse microbiota secondary to gastric atrophy.

**Design—**Gastric colonisation with Altered Schaedler's flora (ASF) and *Hp* were correlated with pathology, immune responses and mRNA expression for proinflammatory and cancer-related genes in germ-free (GF), *Hp* monoassociated (m*Hp*), restricted ASF (rASF; 3 species), and specific pathogen-free (complex IF), hypergastrinemic INS-GAS mice 7 months postinfection.

**Results—**Male mice cocolonised with rASF*Hp* or IF*Hp* developed the most severe pathology. IF*Hp* males had the highest inflammatory responses, and 40% developed invasive gastrointestinal intraepithelial neoplasia (GIN). Notably, rASF*Hp* colonisation was highest in males and 23% developed invasive GIN with elevated expression of inflammatory biomarkers. Lesions were less severe in females and none developed GIN. Gastritis in male rASF*Hp* mice was accompanied by decreased *Clostridum species* ASF356 and *Bacteroides species* ASF519 colonisation and an overgrowth of *Lactobacillus murinus* ASF361, supporting that inflammation-driven atrophy alters the gastric niche for GI commensals. *Hp* colonisation also elevated expression of *IL-11* and

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cancer-related genes, *Ptger4* and *Tgf-β*, further supporting that *Hp* infection accelerates gastric cancer development in INS-GAS mice.

**Conclusions—**rASF*Hp* colonisation was sufficient for GIN development in males, and lower GIN incidence in females was associated with lower inflammatory responses and gastric commensal and *Hp* colonisation. Colonisation efficiency of commensals appears more important than microbial diversity and lessens the probability that specific gastrointestinal pathogens are contributing to cancer risk.

## INTRODUCTION

Gastric adenocarcinoma (GAC) represents the most prevalent form (~95%) of gastric cancer, and is the fourth and fifth most common cancer in men and women, respectively.[1] Unlike the poorly differentiated diffuse-type GAC which often arises without precancerous lesions, a more differentiated intestinal-type GAC is the end stage of lesions progressing from gastritis, atrophy, metaplasia and dysplasia to carcinoma in situ.[23] Among risk factors for intestinal-type GAC, *Helicobacter pylori* (*Hp*) infection is one of the most important risk factors.[4–6] However, of the 50% of the world's population infected with *Hp*, only 1–2% develop gastric tumours,[7] while most infected individuals experience asymptomatic pangastritis.[89] This differential susceptibility to GAC has been attributed, in part, to differences in virulence potential of diverse *Hp* strains,[41011] host genetic polymorphisms involved in immune and inflammatory responses, cellular metabolism, growth and differentiation,[1213] and environmental variables including diet, coinfections and age of *Hp* acquisition.[314]

Recent studies in humans indicate that gastric colonisation by non-*Hp* bacteria, such as *Actinobacteria*, *Bacteroides*, *Firmicutes*, *Fusobacteria* and *Proteobacteria*, many of which normally colonise the lower bowel, could impact the risk for GAC.[15–18] Dyspeptic *Hp* patients treated with acid-suppressive drugs had a significant increase in non-*Hp* bacteria colonising the stomach, and inflammatory cytokine levels associated with greater risk of atrophic gastritis, suggesting that non-*Hp* bacteria colonise the stomach during acid-suppressive therapy and promote gastric carcinogenesis.[1920] This paradigm has been modelled in the *Hp*-infected gerbil with increased gastric atrophy and promotion of GAC when treated long-term with proton pump inhibitors.[21] An altered microbial composition with overgrowth of *Streptococcus*, *Lactobacillus*, *Veillonella* and *Prevotella* has similarly been found in the stomach of *Hp*-infected GAC and dyspeptic patients.[22] *Acinetobacter iwofii*, a bacteria causing nosocomial infection in ventilated patients, induced gastritis, hypergastrinemia, inflammatory cytokine and gastrin production comparable with *Hp* infection in C57BL/6 mice, further suggesting that gastric colonisation by non-*Hp* pathogenic bacteria could promote GAC.[23] As previously shown, normal intestinal flora (IF) hastened the onset and promoted the progression of gastrointestinal intraepithelial neoplasia (GIN),[24] while antimicrobial therapies delayed onset of GIN in *Hp*-infected, and importantly, in uninfected INS-GAS mice.[25] Notably, *Hp*-free INS-GAS mice colonised with IF developed GIN more quickly than germ-free (GF) INS-GAS mice which remained free of GIN through 11 months of age.[24] Similar results were observed in the *K19-Wnt1/C2mE* mouse model of GAC.[26] Together, these data suggest that non-*Hp* bacteria, including

those considered as pathogenic or commensal IF, can colonise the stomach and represent an additional GAC risk, particularly in *Hp*-infected, susceptible individuals.[19,22]

Although evidence supports a role for normal IF in GAC, it is unknown if specific species of IF are required to promote GAC progression or if the mechanism reflects gastric colonisation with diverse IF secondary to *Hp*-mediated gastric atrophy. Using the transgenic, hypergastrinemic INS-GAS mouse model of *Hp*-accelerated gastric carcinogenesis, we evaluated if a restricted microbiota limited to three species of Altered Schaedler's Flora (restricted ASF or rASF), including ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus* and ASF519 *Bacteroides* species, is sufficient to contribute to the GIN incidence after development of gastric atrophy secondary to *Hp* infection.[27,28] Under gnotobiotic conditions, male and female INS-GAS mice that were GF, monoassociated with *Hp* (m*Hp*) or colonised with rASF, rASF*Hp*, IF (specific pathogen-free or SPF with undefined IF), or IF*Hp* were compared for gastric pathology, ASF and *Hp* colonisation dynamics, inflammatory responses in serum and mRNA expression of select proinflammatory and cancer-related genes in gastric tissues.

## MATERIALS AND METHODS

### Experimental design

Animal use was approved by the MIT Committee on Animal Care. Six experimental groups of INS-GAS mice on a FVB/N background (Tg (Ins1-GAS) 1Sbr) included GF (n=32: 15 males and 17 females), rASF (n=27: 15 males and 12 females), IF (n=19: 10 males and 9 females), m*Hp* (n=12: 6 males and 6 females), rASF*Hp* (n=22: 13 males and 9 females) and IF*Hp* (n=24: 15 males and 9 females). Methods for rASF, IF and *Hp* colonisation of INS-GAS mice, husbandry, necropsy, histologic scoring, analysis of serum and gastric mRNA expression levels of cytokines and chemokines, quantitative PCR (qPCR) for ASF and *Hp* colonisation levels, ELISA for serum immunoglobulin responses to *Hp*, and statistical analysis, are provided in online supplementary materials and methods. Because gastritis and development of GIN in the INS-GAS model is male-predominant, the Results section focuses on data from male mice with relevant comparisons made to data from female mice which are more comprehensively presented in online supplementary results. All p values for significant results at $p<0.05$ are presented in the Figure and Table legends.

## RESULTS

### rASF Alone was sufficient to promote gastric pathology in gnotobiotic INS-GAS mice

Compared with GF, male INS-GAS mice colonised with rASF or IF had equivalent, higher median Gastric Histologic Activity Index (GHAI) scores, supported by significant differences from GF mice in all GHAI subfeatures, including mild to moderate gastric corpus inflammation, epithelial defects, oxyntic gland atrophy, epithelial hyperplasia, pseudopyloric metaplasia and dysplasia (figures 1 and 2). Although mild to moderate gastric dysplasia developed in rASF and IF INS-GAS mice, none of them developed GIN (table 1) at the age equivalent to 7 months post-infection (mpi) with *Hp*. GHAI scores for female INS-GAS mice were lower compared with male mice, but differences observed between

groups in male mice were also observed between groups of female mice having different colonisation status (see online supplementary figures S1 and S2). These results indicate that rASF alone, similar to IF, can promote gastric pathology in male and female INS-GAS mice independent of *Hp* infection, however, to a lesser extent in female INS-GAS mice.

### *Hp* accelerated the onset and progression of GIN in rASF and IF colonised INS-GAS mice

Because our previous studies demonstrated that IF and *Hp* synergistically promoted invasive GIN in 80% of male SPF INS-GAS mice by 7 mpi,[24] we next determined whether rASF could also synergistically promote GIN when combined with *Hp* infection. Compared with GF, rASF, IF and m*Hp* INS-GAS mice, rASF*Hp* and IF*Hp*-cocolonised male INS-GAS mice developed the most severe gastric pathology, as reflected by higher GHAI scores (figure 1). As evidence that progression of gastric lesions towards GIN was accelerated in *Hp*-infected INS-GAS mice by cocolonisation with rASF or IF, 69% of male rASF*Hp* and 93% of male IF*Hp* INS-GAS mice developed sufficient dysplasia to be categorised as GIN, whereas GIN was not detected in other groups of mice at 7 mpi (table 1). GIN lesions were characterised by progressive dysplasia including loss of glandular organisation, crowding and atypia with variable globoid dysplasia, a feature characterised by disorderly stratification of proliferating gastric glandular epithelial cells that were expanded by large cytoplasmic mucus vacuoles with nuclear margination (figure 2). Interestingly, both rASF*Hp* and IF*Hp* male INS-GAS mice developed invasive GIN categorised as either intramucosal carcinoma (invasion into the lamina propria or muscularis mucosa) or carcinoma extending into or beyond the submucosal margins. The incidence of invasive GIN was only slightly lower in rASF*Hp* (23%) compared with IF*Hp* (40%)-colonised male INS-GAS mice (p=0.17). Together, these results suggest that colonisation with rASF*Hp* alone was sufficient to promote all GHAI subfeatures comparable with IF*Hp*-colonised male mice. GIN did not develop in any group of female INS-GAS mice (table 1), consistent with prior studies.[102429]

### rASF*Hp* and IF*Hp*-colonised male INS-GAS mice developed robust local gastric tissue and systemic inflammatory responses in serum

Colonisation of male INS-GAS mice with rASF or IF without *Hp* infection caused moderate elevation of mRNA for proinflammatory genes in gastric samples, including *Nos2, Tnf-α, Cxcl1*(*Kc*) and *Ccl2*(*Mcp-1*) (figure 3). Compared with rASF and IF, *Hp* infection alone induced much higher expression levels for all proinflammatory genes evaluated, suggesting that *Hp* is a potent stimulator of gastric inflammatory response. Consistent with the synergistic effect of *Hp* and intestinal micro-biota, expression levels for proinflammatory genes were highest in IF*Hp*-colonised mice, including *IL-17, Nos2, Tnf-α* and *Cxcl1*(*Kc*). rASF cocolonisation with *Hp* was very similar to IF*Hp* in increasing expression of *Tnf-α, IL-17, Ccl2*(*Mcp-1*) *and Ccl3*(*Mip1-α*). Similar patterns of mRNA levels were observed in female INS-GAS mice but at lower magnitudes (see online supplementary figure S3).

Consistent with gastric tissue mRNA expression, serum levels of IL-17, Cxcl1 (Kc), Tnf-α, IL-12 p70 and p40, Ccl2 (Mcp-1), G-CSF, IL-5, CM-CSF and Ccl5 (Rantes) were significantly elevated in response to *Hp* infection in male INS-GAS mice (see online supplementary figure S4). Except for serum levels of IL-17 which were highest in IF*Hp*-

colonised mice, elevated serum levels of all inflammatory mediators evaluated were similar between m*Hp*, rASF*Hp* and IF*Hp*-colonised mice, indicating *Hp* infection was the main cause of elevated serum inflammatory markers. Similar patterns of increased levels of inflammatory mediators in serum were observed in groups of female INS-GAS (see online supplementary figure S4).

### *Il-11* expression was positively correlated with *Hp* infection and gastric atrophy in male INS-GAS mice

Elevated *IL-11* expression in parietal cells has been associated with *Hp* infection and the severity of gastric atrophy in mice.[30] IF colonisation in male INS-GAS mice increased gastric *IL-11* expression by almost fivefold, and m*Hp* male mice had increased mRNA levels for *IL-11* of approximately 15-fold over GF mice (figure 4). *IL-11* expression was similarly elevated 10-fold in mice cocolonised with rASF*Hp* and IF*Hp*. By contrast, female INS-GAS mice had no comparable changes in *IL-11* expression (figure 4), consistent with greater oxyntic gland atrophy in male INS-GAS mice (see online supplementary figure S1).

### *Hp* infection caused major shifts in gastric ASF colonisation

Because gastric colonisation with rASF or IF promoted progression of *Hp*-associated GIN, gastric colonisation with *Hp* and all eight ASF species was quantified using qPCR as previously reported.[31] All three members of rASF (ASF356, ASF361, ASF519) were detected in the stomach of all male and female rASF and rASF*Hp* INS-GAS mice (see online supplementary figure S5) and it was therefore possible to determine total gastric bacterial colonisation levels in the gnotobiotic mice (rASF, m*Hp* and rASF*Hp*) and the potential shift in colonisation as a result of *Hp* infection between the 3 ASF species. The majority of the 8 ASF species were detected in the IF and IF*Hp* mice (see online supplementary figure S6), but total gastric bacteria were not determined in these mice because the microbiota was undefined.

*Hp* colonisation was similar in male and female m*Hp* and rASF*Hp* mice but was lower in IF*Hp* mice of both genders, particularly in female IF*Hp* mice (figure 5). Two of 15 IF*Hp* males and three of eight IF*Hp* females were PCR negative for *Hp* by 7 mpi, but remained seropositive to *Hp* (figure 6) and had gastritis consistent with their experimental cohorts (figure 1; see online supplementary figure S1), indicating *Hp* had colonised but was cleared late in the infection. Remarkably, total gastric bacteria were significantly higher in male compared with female rASF mice and when cocolonised with *Hp*, total bacteria significantly decreased only in rASF*Hp*-colonised male mice (figure 7). *Hp* colonisation was several logs lower than ASF in rASF*Hp* mice, contributing just 0.7% and 0.4% of the total bacterial colonisation levels in male and female rASF*Hp* mice, respectively (figure 8). In male rASF mice, *Bacteroides* species ASF519 predominated in the stomach followed by smaller, similar percentages of *Clostridium* species ASF356 and *L murinus* ASF361. Cocolonisation with *Hp* significantly increased colonisation of ASF361 with corresponding reductions in ASF356 and ASF519. As a percentage of total gastric bacteria (figure 8), emergence of ASF361 to predominate in the gastric niche was even more dramatic in female rASF*Hp* mice, although females had lower absolute numbers of total gastric bacteria compared with male mice (figure 7).

As the gastric and intestinal microbiota of the IF and IF*Hp* mice were SPF but otherwise undefined, it was not possible to determine the total gastric bacterial colonisation levels, but shifts in the absolute colonisation levels between the eight species of ASF had parallels to observations in rASF mice when cocolonised with *Hp* (see online supplementary figure S6). Notably, colonisation of *Clostridium* species ASF356 and *Bacteroides* species ASF519 were decreased in male IF*Hp* compared with IF mice and *Lactobacillus* species. ASF360 and *L murinus* ASF361 increased in response to *Hp* infection. Additionally, *Eubacterium plexicaudatum* ASF492 increased when mice were challenged with *Hp*. Most of these shifts in the balance between ASF observed in male mice were also observed in females (see online supplementary figure S6).

All rASF (see online supplementary figure S7) and eight species of ASF (data not shown) were detected in the caecum of both male and female rASF, rASF*Hp*, IF and IF*Hp* INS-GAS mice, respectively. However, ASF colonisation levels in the cecum were similar between comparable groups of mice with or without *Hp* infection.

### Serologic response to *Hp* correlated with the diversity of IF colonising the stomach

INS-GAS mice experimentally dosed with *Hp* developed robust IgG responses to *Hp* outer membrane antigens (figure 6). IgG responses were highest in IF*Hp* INS-GAS mice, although rASF*Hp* mice had similar promotion of the IgG response compared with m*Hp* mice. Responses were higher in females compared with male IF*Hp* and rASF*Hp* mice, but gender impact was not observed for m*Hp* mice. Th1-associated IgG2a and Th2-associated IgG1 responses followed the same trend as the total IgG response with IgG2a/IgG1 ratios ranging from 1 to 1.7 without differences between groups of mice (data not shown).

### *Hp* infection elevated expression of the prostaglandin *Ptger4* and *Tgf-β* cancer-related genes

Meta-analyses of genome-wide association studies (GWAS) in humans with GAC revealed *Prostaglandin E receptor* 4 (*Ptger4*) and *Zinc finger and BTB domain containing 20* (*Zbtb20*) to be highly associated with GAC.[12,13] Additionally, *FAT tumour suppressor homologue 4* (*Fat4*) and *AT-rich interactive domain-containing protein 1A* (*Arid1a*) were found to be mutated in somatic germ cells of gastric tumours.[13,32] *Tgf-β* has anti-inflammatory and antioncogenic and pro-oncogenic properties[33] and altered gene expression may thus be associated with the incidence of GAC in INS-GAS mice. Therefore, mRNA expression levels of these genes were analysed to determine whether *Hp*, rASF and/or IF are associated with altered gene expression in INS-GAS mice.

Interestingly, male INS-GAS mice had a fourfold higher expression level of *Ptger4* when challenged with *Hp* irrespective of GF, rASF or IF colonisation status, whereas female mice had a threefold higher expression level when cocolonised with rASF*Hp* and IF*Hp* (figure 4). Significant changes (>twofold) in expression of mRNA for *zbtb20*, *Fat4* and *Arid1a* were not detected between GF, rASF and IF mice (figure 4). Similar to expression levels of *Ptger4*, male INS-GAS mice that were colonised with m*Hp*, rASF*Hp* or IF*Hp* had similar levels of *Tgf-β* expression that were fourfold higher than those of male GF, rASF and IF mice. Thus, *Tgf-β* expression was *Hp*-dependent in male INS-GAS mice.

## DISCUSSION

This study focused on a mechanism of IF promotion of GAC secondary to *Hp*-mediated gastric atrophy (achlorhydria) as proposed in recent literature in both humans and animal models.[2223] We demonstrated that gastric colonisation with three species of commensal bacteria was comparable with colonisation with diverse intestinal microbiota in promoting gastritis and dysplasia.[27] Commensal and *Hp* cocolonisation were required for peak inflammatory responses and GIN development. rASF*Hp* colonisation was sufficient for GIN development in males. Lower GIN incidence in females was associated with lower inflammatory responses and lower gastric commensal and *Hp* colonisation. Colonisation efficiency of commensals appears more important than IF diversity and lessens the probability that specific gastrointestinal pathogens are contributing to gastric cancer risk. Consistent with epidemiology studies reporting that the incidence of stomach cancer is approximately twice as high in men compared with women,[1] and that oestrogen appears protective in humans,[34] the male INS-GAS gnotobiotic model reproduced key features of GAC observed in humans with advanced gastric atrophy secondary to *Hp* gastritis.

Even without *Hp* infection, male rASF and IF INS-GAS mice developed gastric pathology similar to the Correa model of progression of chronic gastritis to atrophy and dysplasia in *Hp*-infected humans.[2] Gastric pathology was promoted to a similar extent by rASF and IF colonisation. Although GIN develops spontaneously in older SPF male INS-GAS mice (~20 months),[35] in this study, *Hp* infection in addition to rASF or IF, were required for gastric lesions to progress to GIN within 7 mpi, as has been demonstrated using *Hp* and *Helicobacter felis* in SPF INS-GAS mice.[1035] Notably, colonisation with rASF*Hp* was not only sufficient to promote all GHAI subfeatures comparable with IF*Hp*-colonised male mice, the incidence of invasive GIN was only slightly lower in rASF*Hp* compared with IF*Hp* mice. These results indicate that rASF alone, similar to IF, can promote gastric pathology in INS-GAS mice independent of *Hp* infection; however, when challenged with *Hp*, time required for progression to GIN is accelerated, and GIN becomes invasive.

By 7 mpi, *Hp* colonisation was lost in a few IF*Hp* male and female INS-GAS mice, consistent with spontaneous eradication of *Hp* in humans and with advanced gastric atrophy.[36] Interestingly, female IF*Hp* mice had less severe gastritis and atrophy, lower incidence of GIN, and decreased *Hp* colonisation compared with male IF*Hp* INS-GAS mice. Consistent with less gastric atrophy, female rASF and rASF*Hp* mice had fewer gastric bacteria compared with males. Particularly in mice due to coprophagy, the gastric mucosa is chronically exposed to colonic bacteria, but acidic gastric pH and the relative aerobic environment prevents significant colonisation. As parietal cells are lost due to chronic gastritis, gastric acidity neutralises allowing some aerotolerant colonic bacteria to colonise the stomach. The same phenomenon has been shown in humans with gastric atrophy, presumably from environmental exposure, reflux of intestinal contents into the stomach, and fecal-oral contamination.[37] Recent studies highlight the possibility that gender influences the microbiome of mouse models[38]; it is possible the male predominance of GIN in INS-GAS mice is, in part, related to gender differences in IF and the established protective effect of oestrogen.[3940] Further investigations are needed to decipher the role of sex hormones in

shaping the gastrointestinal microbiota and how this may promote male-predominant GIN in INS-GAS mice.

Although GHAI scores for rASF*Hp* and IF*Hp* male INS-GAS mice were similar, IF promoted a greater inflammatory response reflected by higher proinflammatory gene expression levels induced by IF*Hp* compared with m*Hp* or rASF*Hp* microbiota. The inflammatory response also was associated with elevated adaptive immune responses as IgG responses to *Hp* were highest in IF*Hp* INS-GAS mice. Although the extent of seroconversion to *Hp* does not correlate with severity of gastritis in humans and only serves as evidence of *Hp* infection, it is probable that IgG responses to *Hp* were highest in IF*Hp* INS-GAS mice because recruitment of inflammatory cells to the stomach likely promoted B cell responses to *Hp*.

Consistent with an inverse relationship between the severity of gastritis and *Hp* colonisation, there was an inverse association between microbial diversity and *Hp* colonisation levels. Interestingly, greater microbial diversity of IF was associated with lower *Hp* colonisation compared with rASF colonisation or *Hp* monoassociation, a result consistent with studies using *H felis*-infected gnotobiotic C57BL/6 mice.[41] In addition to the inhibitory effects of inflammation on *Hp*, the microbiota of the IF-colonised mice was SPF, but otherwise undefined, and thus unidentified bacteria may have out-competed and lowered *Hp* colonisation. *L murinus* ASF361 increased in rASF*Hp*-colonised mice; additionally in IF*Hp* INS-GAS mice, there was increased gastric colonisation with *Lactobacillus* species ASF360, *L murinus* ASF361 and *Eubacterium* ASF492 in response to *Hp* infection. These shifts in gastric microbiota were associated with decreased *Hp* colonisation. It has been shown that *Lactobacillus casei* inhibits *Hp* colonisation.[41] Recently, investigators using *H pylori*-infected C57BL mice from two commercial sources noted that mice from one commercial source had significantly higher gastric inflammation scores than the mice from the second source despite similar *H pylori* colonisation in mice from both sources.[42] They attributed these findings, in part, due to differences in gastric colonisation of *Lactobacillus* species ASF360 and ASF361. The mice with more pronounced *H pylori*-induced gastritis had significantly more ASF361, whereas those with less pronounced *H pylori* gastritis had significantly higher numbers of ASF360.[42] They also noted that fewer CDT4 T cells infiltrated in the stomach and IFNγ transcript were reduced in *H pylori*-infected mice pretreated with antibiotics which had altered their gastric colonisation with enteric flora.[42] *Lactobacillus* species present in rASF and IF may have limited *Hp* colonisation in INS-GAS mice. Furthermore, the negative effect of IF on *Hp* colonisation may explain why gastric *Hp* often declines or disappears during progression to atrophic gastritis.[36][43][44] These results align with the view that once *Hp* initiates inflammation and sufficient gastric atrophy develops, eradication of *Hp* spontaneously or by antimicrobial therapy may not inhibit cancer progression, in part, because IF persistently colonise the stomach and promote inflammation.

Although most of these shifts in the balance between ASF observed in male mice were also observed in females, little is known of the significance of changes in specific ASF species, other than to serve as an indicator that IF commensals may find a preferred niche in the stomach damaged by persistent *Hp* gastritis. Although ASF appear to be beneficial to

mice,[31] it is possible they play a role in the formation of nitrosamine, oxygen radicals, other oxidising agents, mutagens or genotoxic compounds, as their colonisation along with *Hp* induces a greater inflammatory and mutagenic response than *Hp* alone.[11204546] Importantly, the host is responding to rASF as male rASF INS-GAS mice had more severe gastritis and elevated expression levels for proinflammatory genes compared with GF mice. Alternatively, ASF, which do not normally cause intestinal inflammation, may have novel virulence genes induced in the achlorhydric stomach that promote gastric colonisation and overt inflammation. It is likely that the transition from the anaerobic lower bowel to the relatively aerobic environment of the stomach requires significant biochemical adaptations in bacteria, such as ASF, that vary in aerotolerance by species. Nonetheless, the effects of *Hp* on ASF colonisation were specific to the gastric environment as cecal ASF colonisation levels were similar between comparable groups of mice with or without *Hp* infection.

Colonisation of male INS-GAS mice with rASF, IF or *Hp* alone caused moderate elevation of diverse proinflammatory genes implicated in human gastritis and gastric cancer,[3] and the addition of *Hp* to rASF or IF colonisation caused dramatic increases in select proinflammatory gene expression in gastric tissues. Consistent with gene expression, serum levels of proinflammatory mediators were significantly elevated in response to *Hp* infection in male INS-GAS mice. *Hp* was a major driver of mRNA expression and serum levels of IL-17, and IF*Hp* male mice had the highest levels, suggesting that select members of IF, such as segmented filamentous bacteria,[47] may have primed TH17 cells to respond to *Hp* infection. *Hp* and IF colonisation also had an effect on gastric tissue expression of *IL-11*, which is expressed by parietal cells and inhibits gastric acid secretion in response to *Hp* infection in mice.[30] *IL-11* expression was highest in m*Hp* male mice which had retained more parietal cell mass compared with rASF*Hp* and IF*Hp* INS-GAS male mice which had the highest scores for gastric atrophy. Monitoring *IL-11* expression over time may, therefore, represent a potential biomarker for determining the extent of gastric atrophy in *Hp*-infected INS-GAS mice and humans. *Tgf-β* expression was *Hp*-dependent as m*Hp*, rASF*Hp* and IF*Hp* INS-GAS mice had similar expression levels. As *Tgf-β* signalling has important pleiotropic roles in inflammation and cancer progression,[33] the increase in *Tgf-β* expression levels detected in gastric tissues from m*Hp*, rASF*Hp* and IF*Hp* INS-GAS male mice supports that *Tgf-β* expression is elevated in response to *Hp* infection and likely has a role in gastric cancer progression.

Little is known about the role of gastric cancer-associated genes identified in GWAS[13] in the INS-GAS mouse model. Interestingly, male and female INS-GAS mice had higher *Ptger4* expression level when challenged with *Hp* irrespective of GF, rASF or IF colonisation. *Ptger4* is involved in the synthesis of prostaglandins, which play important roles in immune modulation.[26] Thus, *Hp*-mediated GAC likely involves prostaglandin synthesis induction via *Ptger4*, consistent with previous observations.[26] By contrast, *Fat4*, involved in cell adhesion, *Zbtb20*, involved in oncogenesis, and *Arid1a*, a regulator of cell development, were not significantly changed (>twofold) in expression, indicating these genes do not have an apparent impact in *Hp*-mediated GAC in INS-GAS mice.

In conclusion, this study highlights the contribution of IF to gastric cancer progression during *Hp* infection. A key finding was that rASF was comparable with IF in promoting the

progression of *Hp* gastritis to GIN in male INS-GAS mice, indicating colonisation efficiency is more important than IF diversity. Further understanding of the complex interaction between host gender, IF and *Hp* could lead to more effective treatment and prevention strategies for GAC.

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## Significance of this study

**What is already known about this subject?**

- Long-term *Helicobacter pylori* infection is associated with gastric atrophy, increased gastric cancer risk and overgrowth of gastric bacteria.
- Overgrowth of gastric bacteria has also been observed during acid-suppressive therapy in *H pylori*-infected patients, and is associated with greater gastritis and more severe gastric atrophy.
- Human gastric microbiota consists of diverse species of bacteria, some of which are considered commensal, and others have been implicated as pathogens.

**What are the new findings?**

- Colonisation with *H pylori* and a restricted microbiota consisting of only three species of commensal bacteria promoted gastric cancer in gnotobiotic male INS-GAS mice to a similar extent as mice colonised with complex microbiota.
- Gastric pathology induced by the restricted microbiota was associated with robust expression of gastric inflammatory and cancer-associated genes, including *TNF-α*, *Ptger4* and *Tgf-β*.
- Gastric colonisation with complex microbiota lowered gastric *H pylori* colonisation in both male and female INS-GAS mice. However, males with a restricted microbiota were colonised with higher total numbers of gastric bacteria than female INS-GAS mice, indicating more severe gastric atrophy facilitates gastric colonisation with microbiota normally found in the lower bowel.

**How might it impact on clinical practice in the foreseeable future?**

- Human patients with advanced gastric atrophy should be monitored for histologic progression and opportunistic colonisation of the stomach with bacteria that may promote gastric adenocarcinoma.

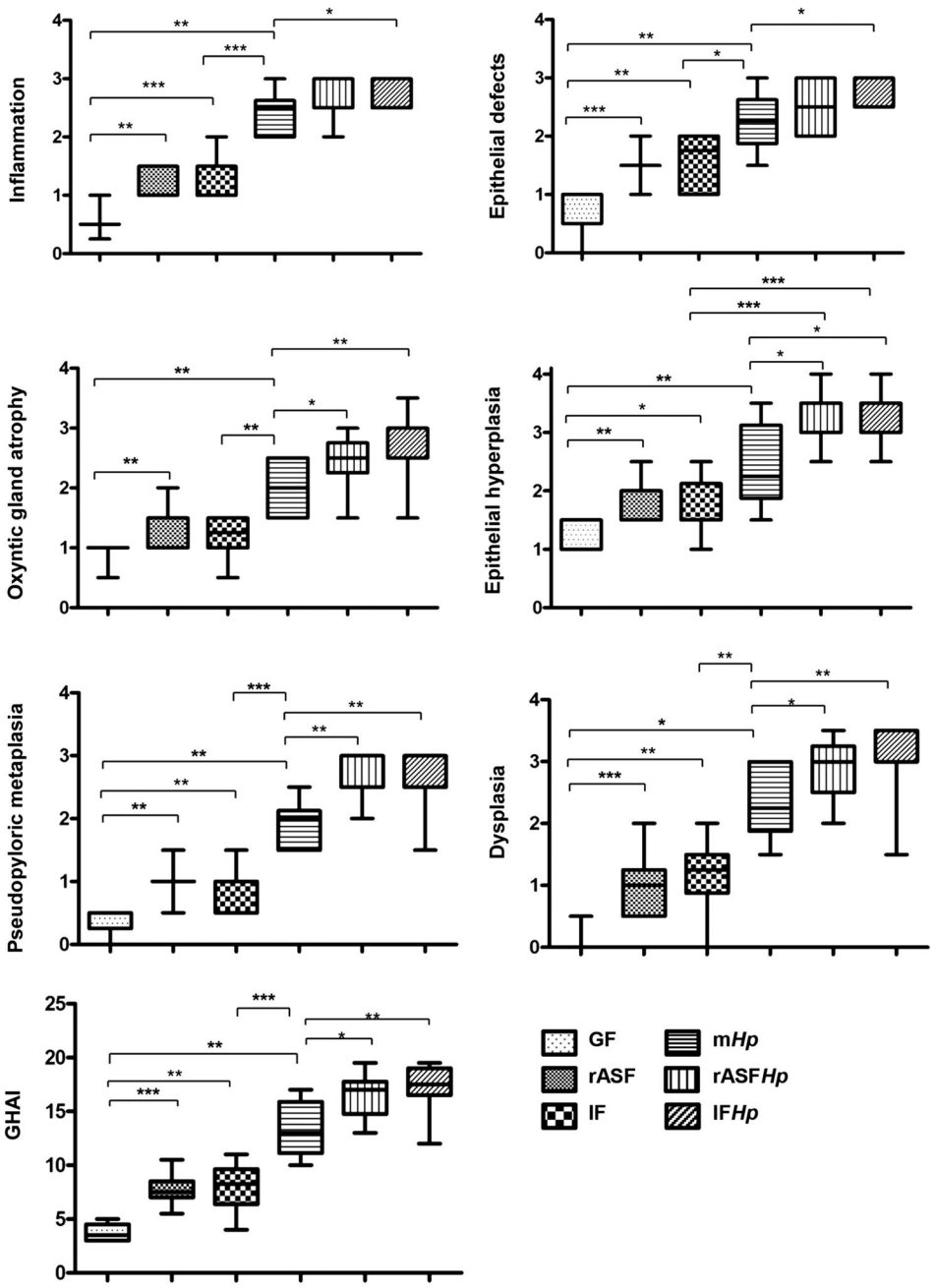


**Figure 1.**

rASF and *Hp* cocolonisation-promoted gastric pathology in male INS-GAS mice to a similar extent as IF. Gastric Histologic Activity Index scores and subfeature scores for gastric lesions in male GF, rASF, IF, m*Hp*, rASF*Hp* and IF*Hp* INS-GAS mice *p<0.05, **p<0.01, ***p<0.001.

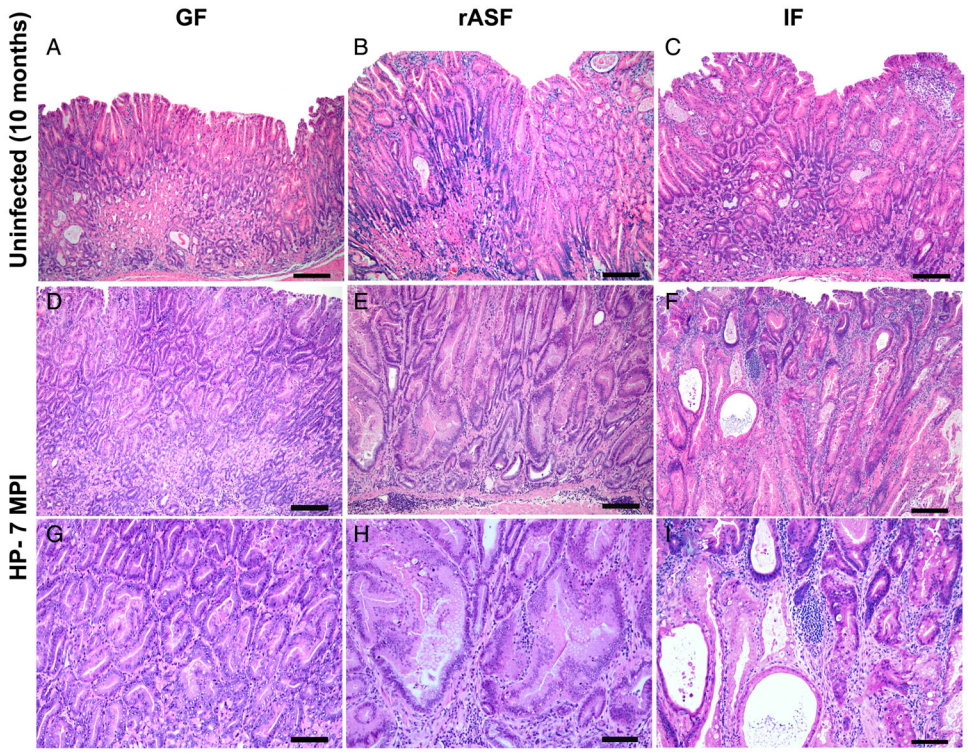


**Figure 2.**

Representative haematoxylin and eosin stained gastric sections from 10-month old male INS-GAS mice. (A) germfree (GF) mice developed sparse mucosal inflammation, mild oxyntic atrophy and mild foveolar hyperplasia, glandular metaplasia and ectasia with minimal dysplasia. (B) restricted ASF (rASF) mice and (C) mice colonised with normal intestinal flora (IF) developed mildly increased severity of all pathomorphological features included in the Gastric Histologic Activity Index scoring system (see Methods and figure 1) as compared with GF mice. INS-GAS mice monoassociated with *Helicobacter pylori* (m*Hp*) at 7 months postinfection (mpi) (D) and (G) at low or high magnification, respectively, developed moderate inflammation, diffuse glandular hyperplasia, severe oxyntic atrophy, pseudopyloric metaplasia and moderate to severe dysplasia including cytological atypia and architectural abnormalities. Mice cocolonised as rASF*Hp* (E) and (H) (at low or high magnification) or IF*Hp* (F) and (I) (at low or high magnification) had more severe pathology including high-grade glandular architectural and cytological abnormalities classified as gastrointestinal intraepithelial neoplasia[48] with invasion into the lamina propria, that is, intramucosal carcinoma (I). Bar values=a to f: 150 μ; G–I: 75 μ.

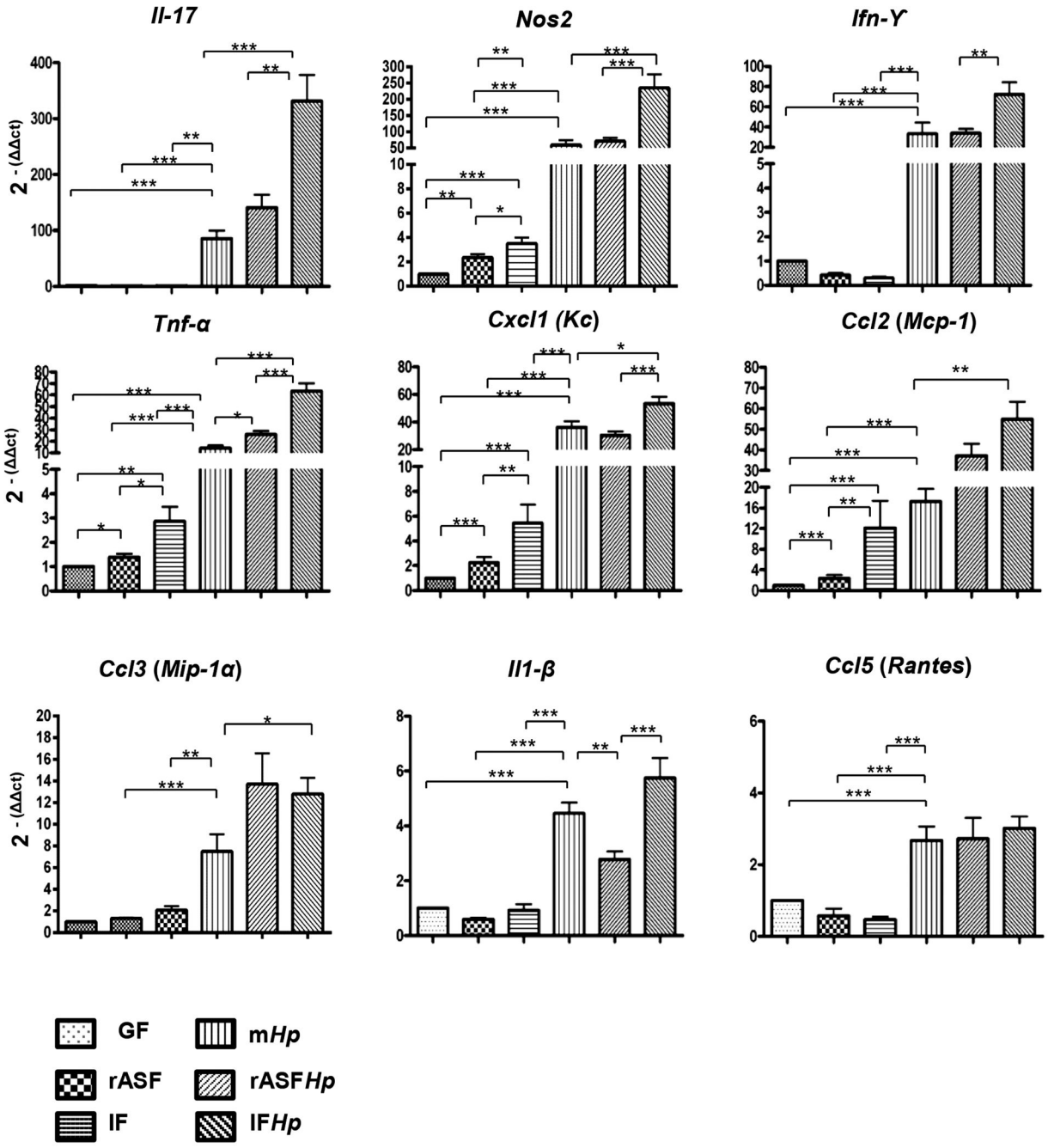


**Figure 3.**

mRNA expression levels of proinflammatory genes in the gastric tissue of male GF, rASF, IF, m*Hp*, rASF*Hp* and IF*Hp* INS-GAS mice at 7 mpi. IF*Hp* INS-GAS mice had the highest inflammatory responses. *p<0.05, **p<0.01, ***p<0.001.

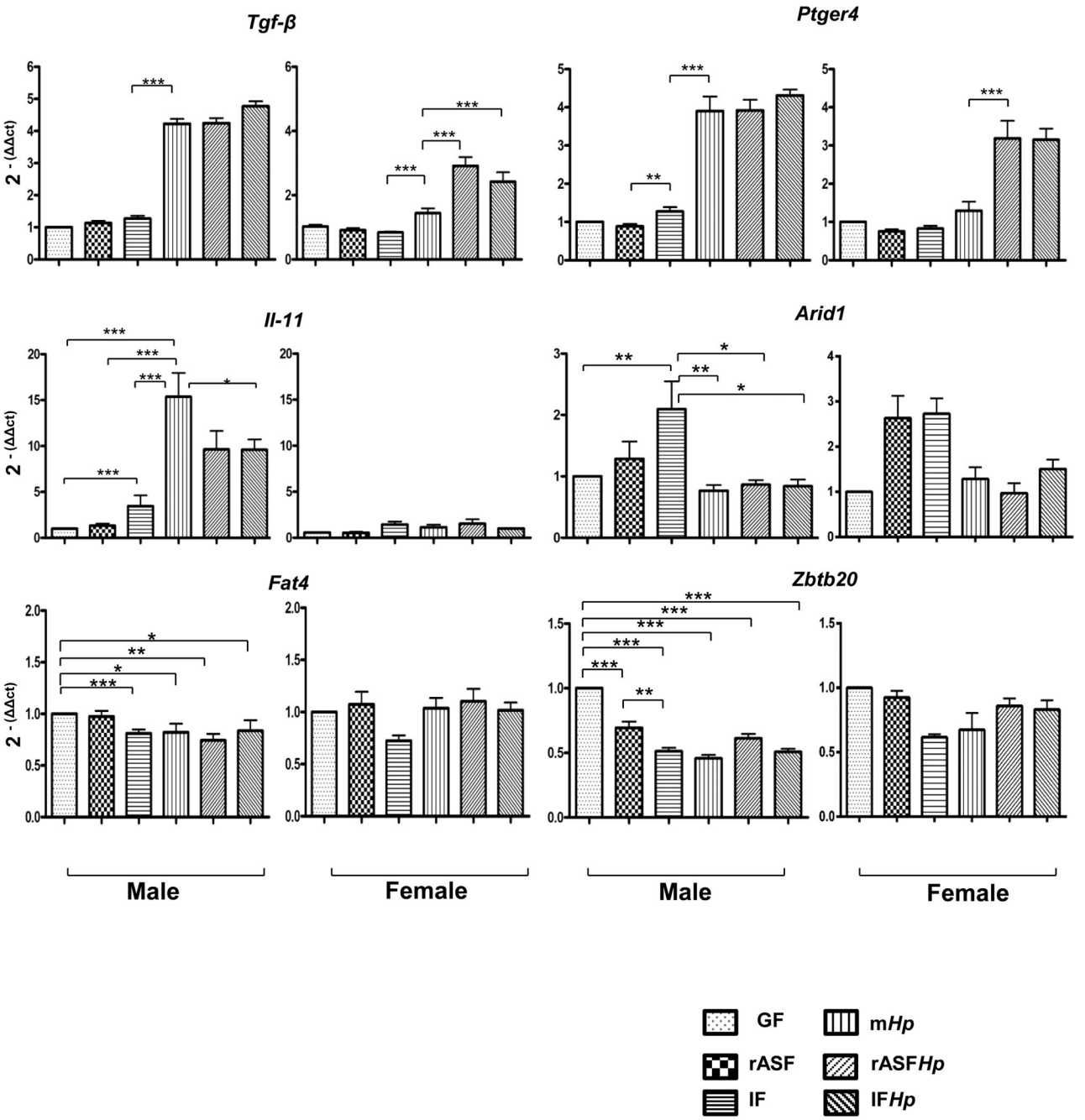


**Figure 4.**

mRNA levels of gastric cancer associated genes in gastric tissue from INS-GAS mice at 7 mpi. Male (left panels) INS-GAS mice had higher expression levels of *Il-11*, *Ptger4* and *Tgf-β* than female (right panels) INS-GAS mice, particularly when colonised by *Hp*. *Hp* colonisation as well as rASF*Hp* and IF*Hp* cocolonisation did not significantly alter the expression of *Arid1*, *Fat4* or *Zbtb20* in male or female INS-GAS mice. *p<0.05, **p<0.01, ***p<0.001.

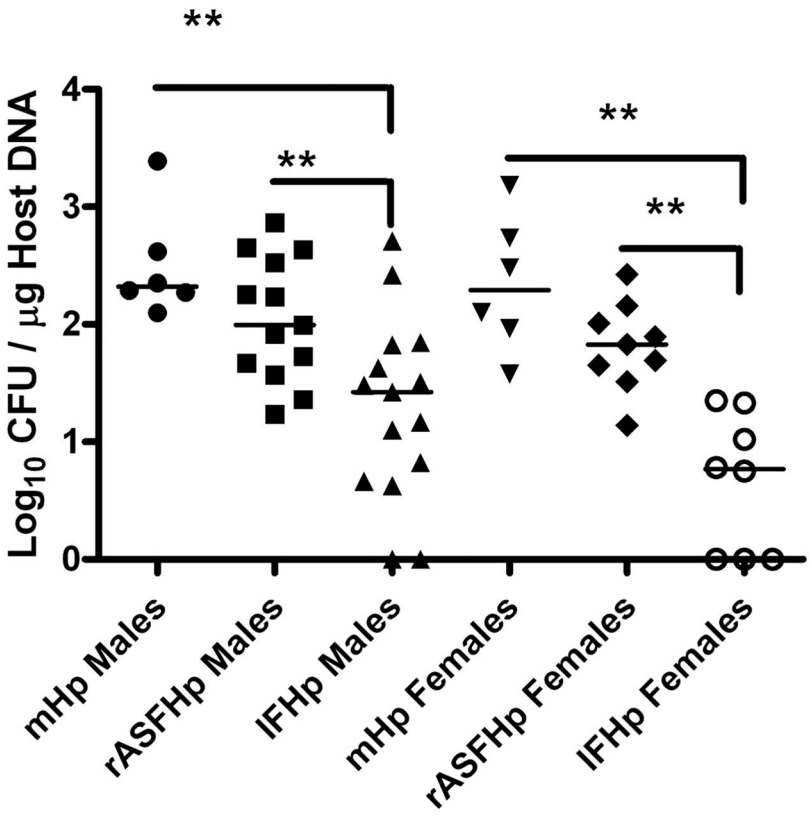


**Figure 5.**

Gastric IF colonisation was associated with decreased *Hp* colonisation in both male and female INS-GAS mice; however, to a greater degree in female INS-GAS mice at 7 mpi. **p<0.01.

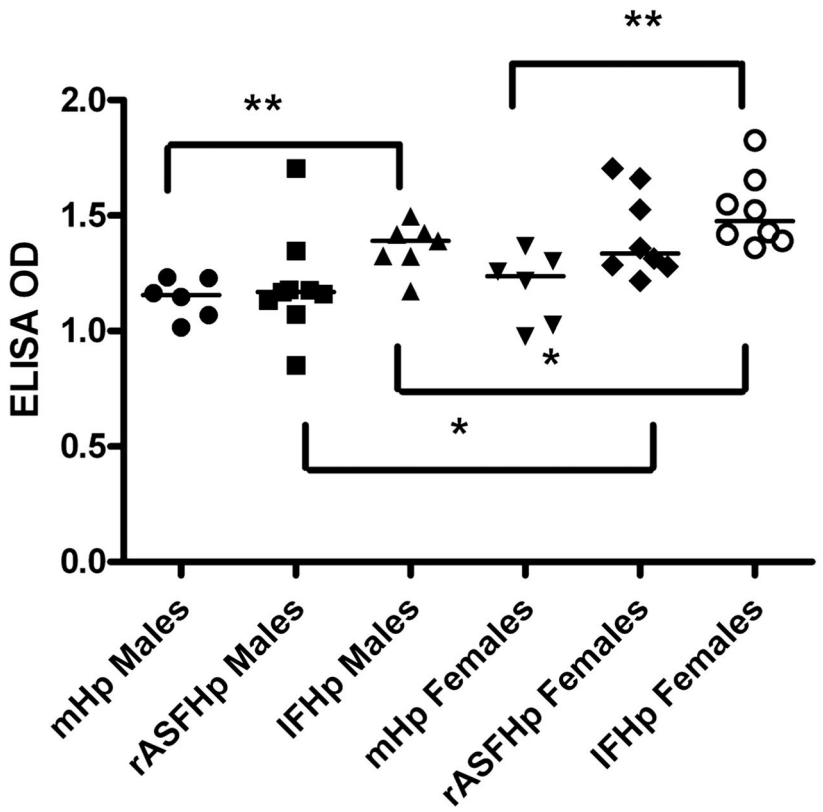


**Figure 6.**

IgG response to *Hp* in male and female m*Hp*, rASF*Hp* and IF*Hp* INS-GAS mice at 7 mpi. The highest IgG responses were associated with the more diverse microbiota of IF*Hp* mice. *p<0.05, **p<0.01.

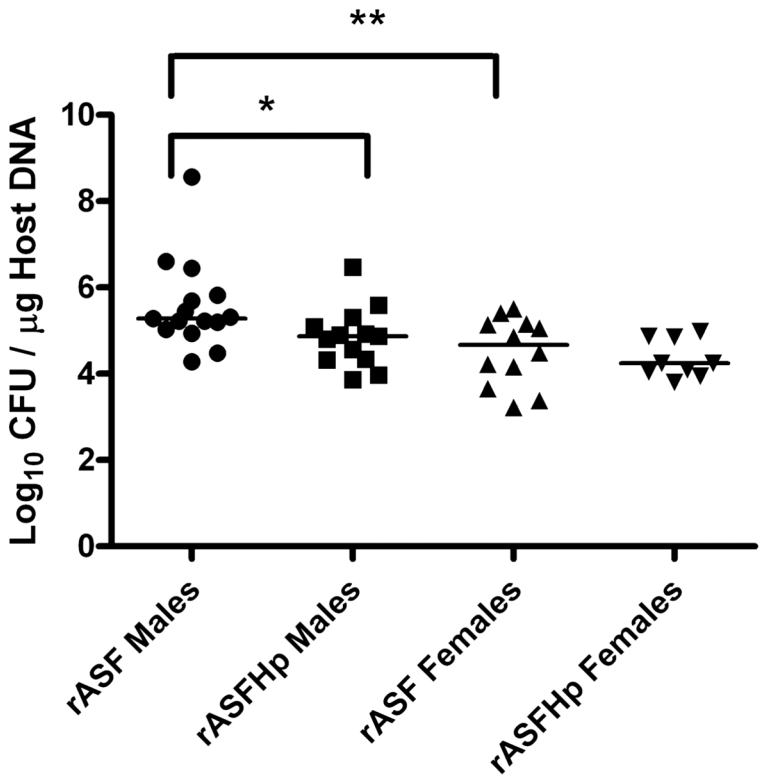


**Figure 7.**

Total gastric bacteria (rASF and *Hp*) in male and female INS-GAS mice colonised with rASF or rASF*Hp*. Male rASF mice had higher bacterial colonisation in the stomach compared with female mice and *Hp* infection reduced these levels in male but not female mice. Total gastric bacteria in male rASF*Hp* mice were higher than in female rASF*Hp* mice at p=0.05. *p<0.05, **p<0.01.

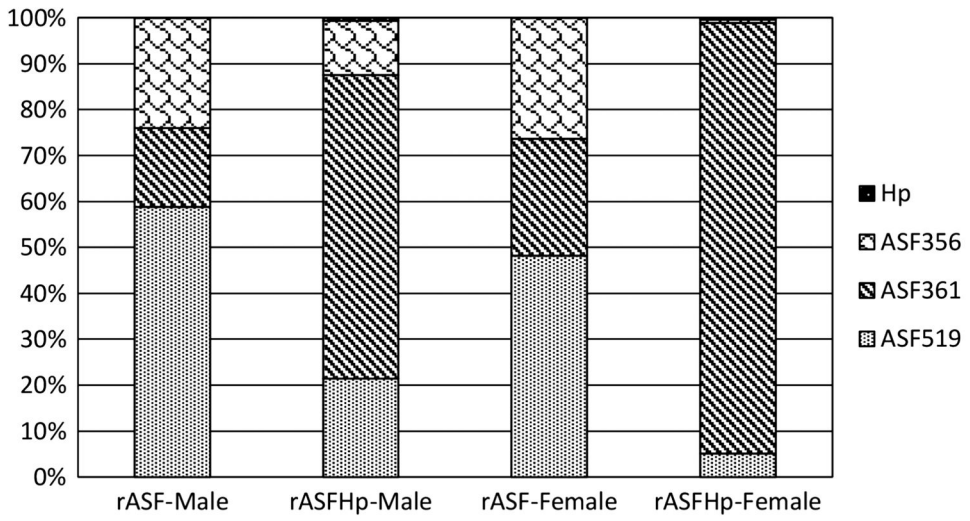


**Figure 8.**

Percentage of total gastric bacteria by bacterial species in rASF and rASF*Hp* male and female INS-GAS mice. *Hp* levels were less than 1% of the total bacteria. In male rASF*Hp* INS-GAS mice, ASF356 and ASF519 were reduced ($p=0.07$, $p<0.001$, respectively). ASF361 colonisation increased significantly as a percentage of total bacteria ($p<0.0001$). Similar shifts in ASF occurred in female mice with *Hp* infection.

**Table 1**

Gastrointestinal intraepithelial neoplasia (GIN) incidence in male and female INS-GAS mice at timepoints equivalent to 7 mpi with *Helicobacter pylori*

| Microbial status | % Of mice with no dysplasia (score=0) | | % Of mice with non-GIN dysplasia* (score 0.5–2.5) | | % Of mice with GIN (high-grade dysplasia) (score=3) | | % Of mice with invasive neoplasms† (score >3) | |
|---|---|---|---|---|---|---|---|---|
| | M (%) | F (%) | M (%) | F (%) | M (%) | F (%) | M (%) | F (%) |
| GF (M=6, F=6) | 17 | 17 | 83 | 83 | 0 | 0 | 0 | 0 |
| rASF (M=10, F=7) | 0 | 14 | 100 | 86 | 0 | 0 | 0 | 0 |
| IF (M=10, F=5) | 10 | 20 | 90 | 80 | 0 | 0 | 0 | 0 |
| m*Hp* (M=6, F=6) | 0 | 66 | 100 | 34 | 0 | 0 | 0 | 0 |
| rASF*Hp* (M=13, F=9) | 0 | 0 | 31 | 100 | 46‡ | 0 | 23‡ | 0 |
| IF*Hp* (M=15, F=9) | 0 | 0 | 7 | 100 | 53‡ | 0 | 40‡ | 0 |

*Non-GIN dysplasia defined as low to moderate grade dysplasia (score 0.5–2.5).

†Invasive neoplasms defined as either intramucosal carcinomas (extending into the lamina propria or muscularis mucosae) or carcinomas extending into or beyond the submucosal margins.

‡similar incidence of high-grade dysplasia in 46% and 53%, and similar incidence of invasive neoplasms in 23% and 40% of rASF*Hp* and IF*Hp* mice, respectively, were significantly higher compared with GF, rASF, IF and m*Hp*-colonised male mice (all $p<0.05$).

F, female INS-GAS mice; GF, germfree; IF, intestinal flora (undefined SPF); M, male; m*Hp*, monoassociated with *H pylori*; rASF, restricted ASF.