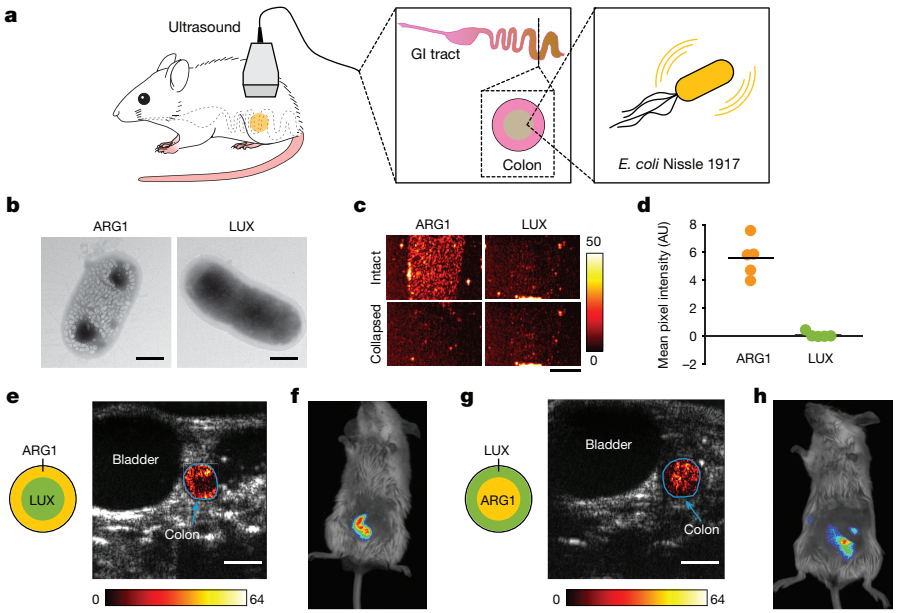

Figure 4 | Ultrasound imaging of bacteria in the gastrointestinal tract. **a**, Diagram of gastrointestinal (GI) imaging experiment. **b**, Representative TEM images of whole ECN cells expressing *arg1* or the *lux* operon. Images were acquired from three biologically independent samples for *arg1* and one for *lux* (approximately 35 cells imaged in each sample) with similar results. **c**, Ultrasound images of a gel phantom containing ECN expressing *arg1* or the *lux* operon. Experiment repeated five times with similar results. **d**, Mean collapse-sensitive ultrasound signal in phantoms containing ECN cells expressing *arg1* or *lux*. Line represents mean. ($P = 0.0007$ using a two-sided heteroscedastic *t*-test, $n = 5$). Cell concentration in **c**–**d** was 10^9 cells ml^{-1}. **e**, Transverse ultrasound image of a mouse whose colon contains ECN expressing *arg1* proximal to the colon wall, and ECN expressing *lux* at the centre of the lumen. **f**, Luminescence image of mouse with the same arrangement of colonic bacteria. **g**, **h**, As in **e** and **f**, but with ECN expressing *arg1* at the centre of the lumen and ECN expressing *lux* at the periphery. Cells are loaded at a final concentration of 10^9 cells ml^{-1}. In **e** and **g**, a difference heat map of ultrasound contrast within the colon region of interest before and after acoustic collapse is overlaid on a greyscale anatomical image. In **f** and **h**, a thresholded luminescence map is overlaid on a bright-field image of the mouse. Scale bars, 500 nm (**b**), 2 mm (**c**) and 2.5 mm (**e**, **g**). *In vivo* imaging experiments were repeated three times with similar results.

than nanostructures formed by cells expressing *arg1* (Extended Data Fig. 5d), and cellular *arg2* contrast was erasable at lower acoustic pressures (Extended Data Fig. 5e). The distinct collapse spectra of the two variants (Extended Data Fig. 5f) allowed *E. coli* expressing *arg1* and *arg2* to be imaged in multiplex using pressure spectrum unmixing (Fig. 3d, e).

After establishing the core capabilities of ARGs *in vitro*, we set out to demonstrate their detectability *in vivo* by imaging ARG-expressing cells in biologically relevant anatomical contexts. One important target for *in vivo* microbial imaging is the mammalian gastrointestinal tract, given the effect of the gut microbiome on the host's health[1,8,9] and the development of gastrointestinal-targeted microbial therapeutics[4,24]. Owing to its location deep inside the body, the gastrointestinal tract is difficult to image using optical techniques. To establish a proof of concept for ultrasonic imaging of microorganisms in this context, we expressed ARGs in a probiotic bacterial strain and assessed the ability of ultrasound to localize this bacterium inside the colon (Fig. 4a) in comparison with bioluminescent imaging. The *E. coli* strain Nissle 1917 (ECN) is a probiotic microorganism capable of colonizing the mammalian gastrointestinal tract[25]. ECN has been used clinically in humans for 100 years to treat enteric infection and inflammatory bowel conditions[25], and is a common chassis for therapeutic synthetic biology[3,5,6,26]. ECN cells transformed with a plasmid expressing *arg1* produced abundant gas vesicles (Fig. 4b) and ultrasound contrast (Fig. 4c, d). For comparison, we transformed ECN cells with the luminescence operon *luxABCDE* (*lux*), which has previously been used to visualize gene expression in microbial populations *in vivo* using bioluminescent imaging[3,6,27]. *lux*-expressing ECN cells produced no ultrasound contrast (Fig. 4c, d).

To establish a proof of concept for ultrasound imaging of ARG-expressing bacteria within the gastrointestinal tract, and to compare the result with bioluminescent imaging, we introduced ECN cells expressing *arg1* or *lux* into the colons of anaesthetized mice. To assess the ability of each modality to resolve the spatial distribution of bacteria within the colon, we injected the *arg1* and *lux* cells into the centre or periphery of the colonic lumen (Fig. 4e–h). Ultrasound images clearly revealed the localization of ARG-expressing ECN cells in the appropriate region of the colon (Fig. 4e, g) at concentrations of 10^9 cells ml^{-1}, which is within the range of certain commensal and therapeutic scenarios, and below the density reached by ECN in gnotobiotic models[21,25]. By contrast, bioluminescent images showed only that the bacteria are present somewhere in the mouse abdomen (Fig. 4f, h). To facilitate visualization of ARG-specific signals, our ultrasound image analysis used background subtraction after gas vesicle collapse, with the resulting contrast overlaid on greyscale anatomical images to show the location of the bacteria within the context of other internal organs. Alternatively, ARG-expressing cells can also be seen in the colon in raw ultrasound images (Extended Data Fig. 6). Contrast from colon-localized *E. coli* was consistent across mice (Extended Data Fig. 7). These results establish the ability of ARGs to make genetically-labelled microorganisms visible noninvasively in deep tissue, and demonstrate the advantage of ultrasound relative to optical imaging in terms of spatial localization within deep organs.

Some degree of burden is expected to accompany heterologous protein expression[28,29]. To assess the burden on ECN cells presented by *arg1*, we characterized their growth, viability, maintenance of reporter expression and release of microcins. We observed that *arg1* expression is generally well tolerated, with some scope for optimization (Extended Data Fig. 8 and Supplementary Note 2).

In addition to the gastrointestinal tract, another emerging application of engineered microorganisms is in antitumour therapies and diagnostics[3,6,30]. To test whether such microorganisms could be imaged with ultrasound, and assess whether ARGs could be generalized to additional species besides *E. coli*, we adapted the genetic construct encoding *arg1* for expression in the attenuated, tumour-homing *S. typhimurium* strain ELH1301 (refs 16, 30), and showed that we could