Intestinal alkaline phosphatase promotes gut bacterial growth by reducing the concentration of luminal nucleotide triphosphates

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Abbreviations: IAP, intestinal alkaline phosphatase; IAP-KO, IAP knockout; LPS, lipopolysaccharides.
ABSTRACT

The intestinal microbiota plays a pivotal role in maintaining human health and well-being. Previously, we have shown that mice deficient in the brush-border enzyme intestinal alkaline phosphatase (IAP) suffer from dysbiosis and that oral IAP supplementation normalizes the gut flora. Here we aimed to decipher the molecular mechanism by which IAP promotes bacterial growth. We used an isolated mouse intestinal loop model to directly examine the effect of exogenous IAP on the growth of specific intestinal bacterial species. We studied the effects of various IAP targets on the growth of stool aerobic and anaerobic bacteria as well as on a few specific gut organisms. We determined the effects of ATP and other nucleotides on bacterial growth. Further, we examined the effects of IAP on reversing the inhibitory effects of nucleotides on bacterial growth. We have confirmed that local IAP bioactivity creates a luminal environment that promotes the growth of a wide range of commensal organisms. IAP promotes the growth of stool aerobic and anaerobic bacteria and appears to exert its growth promoting effects by inactivating (dephosphorylating) luminal ATP and other luminal nucleotide triphosphates. We observed that compared to wild-type mice, IAP-knockout mice have more ATP in their luminal contents, and exogenous IAP can reverse the ATP-mediated inhibition of bacterial growth in the isolated intestinal loop. In conclusion, IAP appears to promote the growth of intestinal commensal bacteria by inhibiting the concentration of luminal nucleotide triphosphates.
INTRODUCTION

Humans and other metazoans have developed a symbiotic relationship with commensal microbiota that provide enormous benefits to the host including vitamin synthesis, food digestion, gut development, immunity and resistance to pathogenic infections (6, 18, 19, 63). Under normal conditions, approximately $10^{14}$ bacteria made up of 300-1000 different species inhabit the human gut (37). Dysbiosis is defined as dysregulation of the normal homeostasis of the intestinal microbiota resulting in an imbalance of the number and composition of intestinal microbes (15, 37). Dysbiosis has been implicated in the pathogenesis of a myriad of disease conditions, including antibiotic-associated diarrhea (AAD), *Clostridia difficile*-associated disease, inflammatory bowel disease (IBD), metabolic syndrome, obesity, and cancer (3, 32, 33, 54, 59). Over the past years, probiosis, prebiosis and fecal biotherapy have emerged as therapeutic approaches for the prevention and treatment of dysbiosis-associated diseases (3, 22, 47, 50, 51). Probiotics are live organisms that provide health benefits and mostly include *Lactobacillus* species, *Bifidobacterium* species, *E. coli*, *Saccharomyces boulardii* and *Trichuris suis* (10, 38, 50, 52, 53). Although clinical trials of probiotics in some instances of dysbiosis have shown promising results, the efficacy of these live organisms appears to be quite limited and there is concern about occasional harmful side-effects (3, 5, 43, 46, 57).

Prebiotics are defined as non-digestible food ingredients that promote the growth of bacteria, stimulate host immunity, prevent pathogenic infections and facilitate host metabolism and mineral absorption (10, 11, 13, 30, 47, 51, 52). Some examples of prebiotics are: xylo-oligosaccharides, galacto-oligosaccharides, oligofructose, lactulose, inulin, and pomegranate extracts (10, 11, 13, 27, 36, 51, 52, 55). Prebiotics may be beneficial to healthy persons, but they
are not generally used in clinically ill patients (45, 60). Up until now, an endogenous factor that regulates the growth of a wide spectrum of gut bacteria has not been identified.

We have previously demonstrated that the brush-border enzyme intestinal alkaline phosphatase (IAP) preserves the normal homeostasis of intestinal microbiota (37). We found that compared to their wild-type (WT) littermates, IAP knockout (KO) mice harbor decreased numbers of both aerobic and anaerobic bacteria. Furthermore, WT mice receiving oral IAP supplementation and an antibiotic were found to rapidly restore the normal microbiota upon termination of antibiotic treatment, thus reducing the antibiotic-induced susceptibility to enteric pathogens such as *Salmonella* Typhimurium and *Clostridium difficile* (1). Based on the above observations, we hypothesized that IAP functions as an endogenous bacterial growth-promoting factor. In the present study we sought to delineate this role of IAP and to define its underlying mechanisms of action. Here, we report that IAP is indeed an endogenous factor that preferentially favors the growth of specific bacterial species, and furthermore, these effects occur due to IAP-mediated reduction of the intestinal luminal concentrations of ATP and probably other luminal nucleotide triphosphates. We believe that orally administered IAP could represent a novel treatment against dysbiosis in humans.
MATERIALS AND METHODS

Chemicals. DNA isolation kit was purchased from Qiagen (Valencia, CA). Bovine IAP, ARL 67156 trisodium salt hydrate, LPS, ATP, ADP, AMP, GTP, CTP, TTP, UTP, p-nitrophenyl phosphate (pNPP), L-phenylalanine, ampicillin and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). ATP analogue 6-\(N,N\)-diethyl-D-\(\beta\)-\(\gamma\)-dibromomethylene ATP (ARL 67156), a selective inhibitor of ecto-ATPases, was also obtained from Sigma. CpG DNA and flagellin were obtained from Invivogen (San Diego, CA). ENLITEN® ATP Assay System Bioluminescence Detection Kit was from Promega (Madison, WI). Isoflurane was obtained from Baxter (Deerfield, IL). dATP, dGTP, dCTP, dTTP, LB broth, LB agar, Brain Heart Infusion (BHI) broth, Brucella agar (5% horse blood) plates were purchased from Fisher Scientific (Pittsburgh, PA). The \(CO_2\) producing gas pack and anaerobic condition indicators were also obtained from Fisher Scientific.

Mice. The construction of C57BL/6 IAP-KO (\(Akp3^{-/-}\)) mice (\(Mus\ musculus\)) has previously been described (41). Heterozygous mice were obtained from the Sanford Burnham Medical Research Institute (La Jolla, CA). These animals were subsequently bred at the Massachusetts General Hospital (MGH) animal facility to create homozygous IAP-KO, heterozygous and wild-type C57BL/6 (WT) littermates. Confirmation of genotype was performed by PCR analysis (41). The mice were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School (Boston, MA) and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Department of Health, Education and Human Services, publication
no. 85e23 (National Institute of Health), revised 1985). The animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at MGH.

Bacterial culture. We observed that normal mouse stool does not contain any streptomycin or ampicillin-resistant aerobic bacteria. Therefore, for our experiments, where we wanted to use such bacteria in an intestinal loop model (see below) we selected representative Gram-negative (Escherichia coli and Morganella morganii) and Gram-positive (Enterococcus faecalis) bacteria from the normal gut flora and selected for streptomycin or ampicillin-resistance. Spontaneous streptomycin-resistant E. coli and M. morganii mutants were isolated by culturing the bacterial sample in LB broth media containing streptomycin (100 μg/ml) followed by sub-culturing the grown bacteria on MacConkey plates containing streptomycin (100 mg/ml). Spontaneous mutations in the rpsL gene render the bacteria streptomycin-resistant and they are phenotypically stable (24). Similarly, ampicillin-resistant E. faecalis was acquired by growing the native E. faecalis in a culture containing ampicillin. To ensure the absence of possible contaminants, the phenotype of each bacterial species was confirmed by the Clinical Laboratory of MGH.

Streptomycin-resistant Gram-negative bacteria (E. coli and M. morganii) were grown in LB-broth and MacConkey-agar plates containing streptomycin (100 μg/ml). Similarly, ampicillin-resistant bacteria (E. faecalis) were grown in LB-broth and LB-agar plates containing ampicillin (100 μg/ml). For stool culture, mouse stool samples were collected fresh directly in BHI media (500 μl) in a microfuge tube, kept on ice, and then vortexed to homogenize the sample, followed by serial dilution and plating on LB-agar and Brucella (5% horse blood)-agar plates. Plates were incubated in ambient air overnight at 37°C for aerobic bacterial growth. For
anaerobic bacterial growth, plates were incubated in a sealed plastic bag containing CO₂ producing gas pack and anaerobic condition indicators (Fisher Scientific) for 3 days at 37°C. Samples were plated on the bench and then placed in the plastic bag. The color change of the anaerobic indicator was strictly monitored. Colony forming units (CFU) were counted and expressed as mean CFU +/- SEM.

The concentrations of IAP targets LPS, CpG DNA and flagellin used in bacterial cultures were 10 μg/ml, 10 μg/ml and 100 ng/ml, respectively. Concentrations of ATP and other nucleotides were 10 mM unless otherwise specified. In an experiment showing IAP-mediated reversal of growth inhibitory effect of ATP (10 mM) on *E. coli*, *Salmonella Typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*, these bacteria were grown in LB-broth containing 10 μl/ml (100 U/ml) of bovine IAP (Sigma) or 10 μl/ml ‘vehicle for IAP’ (50 mM KCl, 10 mM Tris-HCl (pH 8.2), 1 mM MgCl₂, 0.1 mM ZnCl₂, and 50% glycerol, also known as IAP storage buffer) (control group). All aerobic cultures (200 μl) were grown in 8 wells of a 96-well clear-bottom plate at 37°C in a shaking incubator, and absorbance (OD₆₀₀) was determined hourly or as indicated. Bacterial growth in a culture at a definite time-point was calculated as average Absorbance (OD₆₀₀) +/- SD.

In vivo intestinal loop model. WT and IAP-KO mice (n = 5 per group unless indicated otherwise) each weighing approximately 25 g were used. The procedure was done under general anesthesia with inhalational isoflurane at 2% and 1 L/min maintenance flow of oxygen. After a midline incision and exteriorization of the gut, a 5-cm segment of proximal jejunum, distal ileum or colon was carefully tied off at the proximal and distal ends, thus constructing the loop. One animal was used to construct only one loop. When indicated, mice (n = 5 per group) were fasted
for 14 h or 48 h to repress the expression of IAP as previously described (17) followed by construction of the loop. Using a 28-G insulin needle, we injected (total of 100 μl) into the loop approximately 1,000 colony forming units (CFU) of specific bacteria along with other reagents [ATP (100 μM), IAP (100 U/ml), ARL 67156 (10 mM)] as indicated. The abdomen of the mouse was closed and the bacteria were incubated in the isolated loop for 2 h, while the animal was still under anesthesia. The loop was then harvested and the animal was sacrificed. The luminal contents were squeezed off, loop was homogenized and both were plated on selective agar plates and grown overnight at 37°C. Bacterial growth was calculated as fold-increase i.e. the total number of bacteria (CFU) in loop (luminal contents + luminal tissue) divided by the number of instilled bacteria.

**Intestinal alkaline phosphatase (IAP) assay.** IAP activity was determined following the protocol as previously described (37). Briefly, contents of a loop were collected by gentle squeeze, centrifuged, and supernatant was collected. Twenty-five μl of the supernatant (or aqueous IAP solution as a control) were mixed with 175 μl of phosphatase assay reagent containing 5 mM of p-nitrophenyl phosphate (pNPP). Optical density at 405 nm was determined approximately 10 min later when the reaction samples usually turned yellowish due to release of p-nitrophenol. IAP activity is expressed as average units/ml sample +/- SEM.

**Collection of intestinal luminal fluid.** Mice were euthanized and the entire section of intestine was dissected out. Luminal contents were collected by gentle squeezing and then centrifuged at 13,000 x g to pellet the debris and bacteria. The supernatant was collected and *E. coli* bacteria were then added to it and incubated overnight at 37°C.
ATP assay. ATP concentration was measured with the ENLITEN® ATP Assay System Bioluminescence Detection Kit according to the manufacturer’s guideline (Promega).

Oral supplementation of L-phenylalanine. The amino acid L-phenylalanine (Phe) inhibits the enzymatic activity of IAP, and when indicated mice (n = 5 per group) were fed 10 mM L-phenylalanine (Phe) in autoclaved drinking water.

Statistical analysis. Statistical significance between two groups was tested using the two-tailed Student’s t test. Statistical significance between more than two groups was tested using one-way ANOVA (analysis of variance). Pillai’s trace multivariate test was performed for the repeated measures ANOVA and p < 0.05 was considered significant. SPSS (version 13, Chicago, IL, USA) software was used for the statistical analyses.
RESULTS

Bacterial growth is inhibited in the jejunal loop of IAP-KO mice. In order to assess the role of endogenous IAP in a pathophysiologically-relevant manner, we employed an in vivo isolated intestinal loop model system. Under general anesthesia, mice underwent laparotomy and a 5 cm segment of the intestine (jejunum, ileum or colon) was isolated and ligated at both ends to create the loop, with care taken to ensure that the vascular supply to the loop was not compromised. Approximately 1,000 colony-forming units (CFU) of specific bacteria were instilled into the loop by injection and allowed to incubate in vivo for 2 h. To ensure that we could distinguish from the commensal organisms, we instilled selective antibiotic-resistant strains into the loop (streptomycin-resistant *E. coli* and *M. morganii* and ampicillin-resistant *E. faecalis*). Following the incubation period, the loop was harvested, homogenized and plated on the appropriate antibiotic selective plates, and grown overnight at 37°C. Figures 1A and 1B show that growth of both *E. coli* and *M. morganii* was significantly higher in the WT compared to IAP-KO jejunal loop (approximately 1.5-fold differences in growth, p < 0.001 for both species). Similar to the results with these Gram-negative species, the growth of *E. faecalis*, a native Gram-positive inhabitant of the gut, was also significantly higher in the WT compared to IAP-KO mice (Figure 1C). These results suggest that the endogenous IAP exerts a global-type growth promoting effect on these commensal gut bacteria.

Inhibition of endogenous IAP reduces bacterial growth. To further delineate the impact of the endogenous IAP enzyme on luminal bacterial growth, we examined the effects of either silencing IAP expression or inactivating the IAP protein. We have previously shown that
endogenous IAP expression is suppressed in rodents during starvation (14, 17). Accordingly, we fasted mice on a water only diet for up to 48 h and compared the impact on *E. coli* growth in the intestinal loop to normally fed mice. Figure 1D shows the progressive inhibitory effects of fasting on the growth of *E. coli* in a jejunal loop. Compared to control fed animals, *E. coli* growth was significantly lower under 14 h and 48 h fasted condition [3.09±0.09 (fed) vs. 2.35±0.12 (14 h fasted) vs. 1.93±0.07 (48 h fasted) fold increase; p = 0.001 (fed vs. 14 h fasted), p < 0.001 (fed vs. 48 h fasted)]. As expected, jejunal IAP levels were reduced by 70% during fasting (Figure 1E).

We next investigated the effects of inactivating the endogenous IAP enzyme via treatment with oral L-phenylalanine (Phe) (14). Prior to isolating the intestinal loop, mice received 10 mM L-Phe in their drinking water for 48 h. Compared to the mice receiving no Phe, *E. coli* growth was significantly reduced in the jejunal loop of mice receiving Phe (2.61±0.10 vs. 1.53±0.12 fold increase, respectively; p < 0.001) (Figure 1F). Similar reduction of *E. coli* growth was observed in the ileal loop (2.72±0.14 vs. 1.53±0.07 fold increase, respectively; p < 0.001) (Figure 1G), as well as in the colonic loop (2.65±0.12 vs. 1.74±0.07 fold increase, respectively; p < 0.001) (Figure 1H). In order to further confirm the role of the IAP enzyme we next instilled exogenous IAP into a jejunal loop of IAP-KO mice. We added 100 U of IAP or the vehicle control along with the bacteria into a jejunal loop of IAP-KO mice. As predicted, we observed a significant increase in *E. coli* growth when exogenous IAP was added to the intestinal loop (2.61±0.12 vs. 1.63±0.07 fold increase, respectively; p < 0.001) (Figure 1I).
The intestinal luminal fluid from IAP-KO mice inhibits bacterial growth. The above experiments established that IAP functions as a bacterial growth promoter within the intestine. We have previously shown that IAP does not directly affect the growth of bacteria (37) and therefore, we hypothesized that IAP must exert its effects through some indirect mechanism, likely by modulating one or more of its known target molecules within the luminal fluid. To determine whether the luminal fluid itself is capable of promoting bacterial growth we examined the bacterial growth in vitro upon incubation with luminal fluids from WT and IAP-KO mice. The mice were euthanized and luminal fluid from the entire small intestine was gently squeezed out and collected. *E. coli* bacteria were then added to the luminal fluid and incubated for 2 h at 37°C. Figure 1J shows that *E. coli* growth was approximately two-fold higher in the luminal fluid obtained from WT compared to IAP-KO mice. We then collected luminal fluids from proximal small intestine (PSI), distal small intestine (DSI) and colon, and tested the growth of *E. coli* (Figure 1K) and *Salmonella* Typhimurium (Figure 1L), comparing the fluids from WT and KO mice. The results indicate that fluids from all these intestinal segments contain the growth regulating properties.

ATP inhibits the growth of stool aerobic bacteria in vitro. The prebiotic property of the WT luminal fluid along with the absence of any direct effects of IAP on bacterial growth indicated that IAP most probably inactivates the inhibitory effect of one or more of its target molecules on bacterial growth. Accordingly, we tested the growth of mouse stool aerobic bacteria in the presence of high concentrations of 4 known IAP targets: LPS, CpG DNA, flagellin and ATP (9, 40). Figure 2A shows that LPS (10 μg/ml), CpG DNA (10 μg/ml) and flagellin (10 ng/ml) had no effect on the growth of aerobic stool bacteria. However, 10 mM ATP
caused an approximate 50% reduction in bacterial growth. ATP significantly attenuated bacterial growth (repeated measures ANOVA: effect of time: $F_{(5, 30)} = 127.09$, $P$ value < 0.001; between groups differences: $F_{(4, 36)} = 3.99$, $P$ value < 0.01; interaction of time and group: $F_{(20, 132)} = 1.58$, $P$ value = 0.07; Tukey’s post hoc test: $P$ value < 0.05). Bacterial growth at the final time point is depicted in Figure 2B.

We then determined the dose-effects of ATP on a lower inoculum of stool aerobic bacteria and found that ATP inhibited growth even at lower concentrations and such inhibition was dose-dependent (repeated measures ANOVA: effect of time: $F_{(3, 68)} = 586.91$, $P$ value < 0.001; interaction of time and group: $F_{(27, 210)} = 4.53$, $P$ value < 0.001; Tukey’s post hoc test: $P$ value < 0.001 for 400, 800, 1600 µM of ATP) (Figure 2C). The highest effect of ATP on bacterial growth was evident after 8 hours of incubation (Figure 2D). As expected, we also observed the dose-dependent inhibitory effect of higher concentrations (mM) of ATP on the growth of higher inoculum of stool aerobic bacteria (data not shown).

We next determined the effects of ATP derivatives on the stool aerobic bacterial growth. The ATP derivatives ADP, AMP and phosphate exerted little or no inhibition on the growth of the intestinal aerobic flora (Figure 2E-F). We also investigated the effects of the ATP derivative adenosine and observed that adenosine has no effect on stool bacterial growth (data not shown).

**IAP reverses the inhibitory effect of ATP on aerobic bacterial growth.** The above data established that ATP can inhibit the growth of mouse stool aerobic bacteria *in vitro*. We next studied whether IAP would be able to reverse these inhibitory effects of ATP, and found that, indeed, IAP is able to reverse the effects of ATP (10 mM) in a dose-dependent manner (repeated measures ANOVA: effect of time: $F_{(2, 77)} = 287.97$, $P$ value < 0.001; between groups differences:
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\[ F_{(5, 78)} = 5.10, P \text{ value} < 0.001; \text{ interaction of time and group: } F_{(10, 156)} = 15.21, P \text{ value} < 0.001; \]

Tukey’s post hoc test: P value < 0.05 for 50 U/ml of IAP and P values < 0.01 for 100 and 200 U/ml of IAP) (Figure 2G-H). These data suggest that IAP may exert its growth promoting effects by blocking the growth inhibitory effects of its target molecule ATP.

ATP inhibits the growth of stool anaerobic bacteria in vitro. The aerobic bacterial population represents only a minor portion of the total gut bacterial population, most of which is anaerobic. Therefore, we sought to determine the effects of ATP and other IAP targets on the growth of mouse stool anaerobic bacteria. We grew the stool bacteria in the presence of ATP (10 mM), LPS (10 \( \mu \text{g/ml} \)), CpG DNA (10 \( \mu \text{g/ml} \)) or flagellin (100 ng/ml) under anaerobic conditions for 72 h at 37°C. In a manner similar to aerobes, we found that the growth of anaerobes was specifically inhibited by ATP (p < 0.01), whereas other IAP targets had no impact on the bacteria (Figure 3A).

We then determined the dose response effects of ATP (Figure 3B) and found that the anaerobes were particularly sensitive to the inhibitory effects of ATP (p < 0.001). We found that 100 \( \mu \text{M} \) ATP caused 50% inhibition of anaerobic bacterial growth (Figure 3B), whereas 1.2 mM ATP completely inhibited the bacterial growth and higher concentrations of ATP killed these bacteria (data not shown). We next investigated the effects of the ATP derivatives ADP, AMP, and phosphate on the growth of these anaerobes and found that, like aerobes, the ATP derivatives at 10 mM concentration did not have any significant impact on the growth of stool anaerobic bacteria (Figure 3C).
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IAP reverses the inhibitory effect of ATP on anaerobic bacterial growth. The above data indicates that ATP has profound growth inhibitory effects on mouse stool anaerobes. We next studied whether IAP could reverse the effects of ATP. We treated the anaerobes with 5 mM ATP in the presence of different concentrations of IAP and found that IAP reverses the growth inhibitory effects of ATP in a dose-dependent manner (Figure 3D).

All nucleotide triphosphates inhibit bacterial growth. The inhibitory effects of the nucleotide ATP on bacterial growth raised the question whether other nucleotides (hydrolyzed products of luminal DNA and RNA) have any effect on bacterial growth. We observed that 10 mM nucleotides (ATP, GTP, CTP, TTP and UTP) as well as deoxynucleotides (dATP, dGTP, dCTP and dTTP) inhibit aerobic bacterial growth and these effects can be reversed by IAP (Figures 4A-J). Similar to aerobes, these nucleotides and deoxynucleotides at 10 mM concentration also inhibited or killed the stool anaerobes and the effects were reversed by IAP (Figure 4K).

ATP differentially inhibits the growth of specific bacteria. We were interested to know whether the growth-inhibitory effects of ATP were similar for all bacterial species. Accordingly, we exposed 2 Gram-positive and 2 Gram-negative bacterial species to different concentrations of ATP (1-20 mM). We observed that ATP slightly inhibited the growth of the Gram-negative species *E. coli* (Figure 5A) and *S. Typhimurium* (Figure 5B), but had more profound inhibition on the growth of the Gram-positive species, *S. aureus* (Figure 5C) and *L. monocytogenes* (Figure 5D). Growth inhibition by ATP was greatest with *L. monocytogenes > S. aureus > E. coli > S.*
These data indicate that ATP has variable effects on the growth of different bacterial species, and seems to affect Gram-positive bacteria more than the Gram-negative bacteria. As expected, IAP (200 U/ml) reversed the inhibitory effects of ATP (10 mM) on the growth of all these bacterial species (Figure 5E-L).

ATP derivatives do not exert significant effects on the growth of selective bacterial species. We determined the effects of ATP derivatives on the growth of \textit{E. coli}, \textit{S. Typhimurium}, \textit{S. aureus} and \textit{L. monocytogenes}. It is apparent that the growth of these bacterial species was not significantly affected by the ATP derivatives except 10 mM ADP inhibited the growth of \textit{S. aureus} by 50% (p < 0.05) (data not shown).

All nucleotide triphosphates preferentially inhibit Gram-positive bacteria. We examined the effects of all nucleotide triphosphates and deoxynucleotides on the growth of Gram-negative \textit{E. coli} and \textit{S. Typhimurium}, and Gram-positive \textit{S. aureus} and \textit{L. monocytogenes}. It is evident that these nucleotides (10 mM) had minimal growth-inhibitory effects on the Gram-negatives (Figure 6A, B); however, they substantially inhibited the growth of the Gram-positive bacteria (Figure 6C, D). The growth-inhibitory effects of all nucleotide triphosphates were reversed by IAP (200 U/ml) treatment (Figure 6A-D).

ATP inhibits bacterial growth \textit{in vivo}. The data presented above established that ATP and other nucleotide triphosphates inhibit bacterial growth \textit{in vitro}. In order to define the
physiological role for ATP and other nucleotide triphosphates on bacterial growth \textit{in vivo}, we focused on determining the effects of ATP in the intestinal lumen. We determined the ATP concentrations in the small intestinal luminal fluids of WT and IAP-KO mice. We observed that compared to WT animals, there was nearly 10-fold more ATP in the intestinal luminal fluid of KO animals (Figure 7A). Fasting silences IAP and hence we examined the concentration of ATP in fasted animals and found that ATP concentration increases approximately 4-fold in the fasted (14 h) animals compared to the fed animals, consistent with the fact that IAP functions to dephosphorylate ATP (Figure 7B). We then determined the concentrations of ATP in the loops of WT vs. IAP-KO mice. Figure 7C shows that compared to WT jejunal loop, ATP concentration was approximately 4.3-fold higher in the IAP-KO jejunal loop. We next directly tested whether IAP destroys ATP \textit{in vivo}. We instilled 100 U IAP into the jejunal loop in IAP-KO mice and observed that compared to the control animals receiving no IAP, the ATP concentration was significantly lower in the mice receiving IAP (approximately 80% reduction) (Figure 7D).

We next sought to determine whether exogenous ATP would inhibit bacterial growth within the context of the intact gut luminal environment. To prevent the destruction of exogenous ATP by the endogenous IAP, we fed WT mice 10 mM Phe in the drinking water for 48 h. We then instilled 100 μM ATP in the loop followed by \textit{E. coli}. We observed that compared to control mice receiving no ATP, \textit{E. coli} growth was significantly lower in mice receiving ATP (1.57±0.07 vs. 1.12±0.05, respectively; p < 0.001) (Figure 7E). To independently confirm this inhibitory effect of ATP on bacterial growth \textit{in vivo}, we instilled into the loop an ecto-ATPase inhibitor ARL 67156 (10 mM), which is known to increase the ATP levels by inhibiting its hydrolysis by ecto-ATPases of CD39 family (31). Again, we observed that compared to mice receiving no ARL 67156, mice receiving ARL 67156 had a significant decrease in bacterial
growth (2.71±0.10 vs. 1.68±0.06, respectively; p < 0.001) (Figure 7F). Taken together, these data indicate that ATP inhibits bacterial growth in vivo and that IAP exerts its growth promoting effects by hydrolyzing ATP and probably other nucleotide triphosphates.
DISCUSSION

It has become clear in recent decades that intestinal dysbiosis plays a critical role in the pathogenesis of many diseases (3, 32, 33, 54, 59). A variety of different factors can be responsible for dysbiosis and include antibiotics, psychological and physical stress, diets rich in protein, simple sugar/refined carbohydrates, fat, or fructose, chemotherapy, radiation treatment, and intestinal infections (7, 15, 16, 42). Probiotics, prebiotics, synbiotics and fecal biotherapy have emerged as therapies against dysbiosis, albeit with limited success (3, 22, 47, 50, 51). Prebiotics are known to modify and enhance the gut functionality, (10, 11, 13, 30, 47, 48, 52) exerting their health benefits via alterations in the composition and/or activity of the intestinal microbiota. In the present study we provide evidence that the brush-border enzyme intestinal alkaline phosphatase (IAP) promotes the growth of commensal bacteria. IAP exerts a number of beneficial effects on the gut, including the maintenance of local gut immunity (8), enhancing barrier function, reducing excessive inflammation, and inhibiting metabolic endotoxemia. (4, 12, 14, 21, 26). The present study delineates another key beneficial impact of the IAP enzyme, promoting the growth of the commensal flora.

We have demonstrated the growth promoting effects of IAP in different in vivo settings using an isolated intestinal loop model. We compared commensal bacterial growth in high vs. low IAP environments by utilizing WT and IAP-KO, as well as fasted and fed mice. In addition, luminal IAP levels were altered by either directly adding exogenous IAP or by inhibiting the luminal IAP activity by adding its inhibitor L-phenylalanine (Phe). Our data indicate that IAP is able to regulate a wide range of commensal bacteria, Gram positives, Gram negatives, aerobes and anaerobes.
We have focused on determining the mechanisms by which IAP exerts its bacterial growth promoting effects. We have previously demonstrated *in vitro* that IAP does not exert any direct growth-promoting effect on specific aerobic bacterial species, such as *E. coli*, *S. Typhimurium*, *S. aureus* or *L. monocytogenes* (37). This observation led us to hypothesize that IAP must work through an indirect mechanism, likely by blocking one or more of its target molecules that otherwise inhibits bacterial growth (37). Accordingly, we tested the effects of known IAP targets and found that only ATP to exhibit an inhibitory impact on bacterial growth. More importantly, these effects of ATP were completely blocked after incubation with IAP, suggesting that IAP works by inactivating ATP. It is interesting to note that the magnitude of the effects of extracellular ATP varies from one bacterial species to another. Where 10 mM ATP had minimal effects on the growth of the Gram-negatives *E. coli* and *S. Typhimurium*, the same concentration of ATP dramatically inhibited the growth of the Gram-positive species, *S. aureus* and *L. monocytogenes*. Atarashi et al. demonstrated that intestinal luminal ATP originates from both dead mammalian cells as well as the bacteria themselves (2) and Ivanova et al. described that Gram-negative bacteria in general, secrete more ATP compared to Gram-positive bacteria (20), which might be the reason why the Gram-negative bacteria are relatively resistant to extracellular ATP. Although the precise mechanism by which ATP inhibits bacterial growth is beyond the scope of this study, it is worth noting that the extracellular ATP-mediated lysis of bacterial cells has been previously reported (62). Since the amount of ATP increases with higher bacterial count and turnover, we speculate that the growth inhibitory effects of ATP could be a mechanism by which bacteria self-regulate their population, avoiding overgrowth that would be detrimental to the host in which they reside, as well as to the growth of their bacterial symbiotic
partners. Figure 8 is a schematic overview of the growth promoting role of IAP in the GI luminal environment and the proposed mechanism.

IAP is a brush border enzyme that is exclusively expressed in villus-associated enterocytes and it is therefore considered a marker for enterocyte differentiation (17). Much recent progress has been made in delineating the physiological and pharmacological properties of the IAP enzyme. IAP has the ability to dephosphorylate a variety of bacterial and host-derived ligands (LPS, CpG DNA, flagellin, UDP), each of which works through a specific receptor to exert its inflammatory impact on target cells (8, 39). Here we show that IAP reverses the growth-inhibitory effects of various nucleotide triphosphates indicating that these nucleotides are also IAP targets (see Figures 2-7), and IAP probably destroys these targets by dephosphorylation (phosphohydrolysis). IAP has been shown to play an important role in fat absorption, and it has been established that the IAP-KO mice are obese when compared to their WT littermates (41). Furthermore, IAP has been found to act as a gut mucosal defense factor and its expression is influenced by exposure to bacteria (4, 14, 25). IAP is also involved in the maintenance of normal gut barrier function (12, 21).

Pharmacologically, IAP has been found to be an effective therapy against various human as well as animal disease models including IBD, necrotizing enterocolitis, gram-negative sepsis, C. difficile and S. Typhimurium infections, and metabolic syndrome (1, 21, 34, 44, 56, 58, 61). Clinical trials using bovine IAP against colitis have shown that IAP has no or minimal side-effects (34).

Recently, we have shown that oral supplementation with IAP prevents high fat diet-associated metabolic syndrome in mice (21). We postulated that IAP detoxifies luminal
lipopolysaccharides (LPS) and therefore less LPS is available to be bound to fatty acids (chylomicron) and be translocated to systemic circulation thus preventing metabolic endotoxemia and consequently the metabolic syndrome. However, it is also possible that more chronic alterations in the flora and/or levels of the extracellular nucleotide triphosphates caused by IAP contribute to the prevention of obesity and the metabolic syndrome.

Here we show that the luminal ATP and other nucleotide triphosphates play a critical role in preventing the bacterial growth. It is likely that the nucleotide triphosphates represent just one factor in the overall regulation of the commensal bacterial population within the gut, working in concert with other endogenous factors, e.g., antibacterial peptides (29, 49). The idea that extracellular nucleotide triphosphates inhibit bacterial growth in the intestine raises the question of whether these molecules play a similar role in other sites, e.g., the mouth, vagina, urinary and respiratory tracts. Some associations have been noted between ATP and certain urinary and respiratory infections (28, 35), but a role in terms of regulating bacterial growth has not been previously reported.

In addition to the intestinal AP, mammalians express other AP forms, including placental, germ cell, neutrophil, and tissue non-specific (liver/bone/kidney) (25). Furthermore, within the mouse intestine, three different IAP isozymes exist (Akp3, 5, and 6). All of the AP enzymes share significant structural homology as well as functional similarities, but it will be of interest in the future to determine whether these other forms of AP also have the capability to promote bacterial growth. ATP is also a known target of ecto-ATPases (23), which would be expected to be intact in the IAP-KO mice. As ATP concentrations are increased in IAP-KO mice, it could be concluded that ecto-ATPases are less efficient than IAP in destroying/inactivating ATP. Further
work will be needed to determine whether other ecto-nucleotidases play any role in regard to regulating the gut microflora.

In conclusion, although caution should always be taken extrapolating mouse studies to humans, the present data indicate that IAP functions as an endogenous promoter of commensal bacterial growth, likely through an inhibition of luminal ATP and other nucleotide triphosphates. As such, we believe that these findings lay the groundwork for developing IAP-based therapies in clinical settings of dysbiosis, either as a supplement or through a diet-related increase in endogenous levels. The concept of using an endogenous enzyme as a supplement to promote commensal bacteria is not only novel, but could also represent a simple and safe way to impact the millions of people world-wide who are at risk for gut-derived infections and conditions associated with dysbiosis.
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Figure Legends

Figure 1: Lower levels of endogenous IAP reduce bacterial growth. Laparotomy was performed on mice under general anesthesia, a 5-cm loop was constructed, and approximately 1,000 CFU of a specific bacterial species were instilled by injection (100 μl) into the loop. After 2 h, the loop was dissected out, homogenized and plated on selective media for overnight growth at 37°C (see Materials and Methods for details). Bacterial growth in the jejunal loops of WT and IAP-KO mice: (A) Growth of *E. coli* (n = 10 in each group); (B) Growth of *M. morganii* (n = 5 in each group); (C) Growth of *E. faecalis* (n = 5 in each group). (D) Growth of *E. coli* in the jejunal loop of animals fasted for 14 and 48 h. (E) IAP levels in the jejunal loops of animals fasted for 14 and 48 h. Growth of *E. coli* in the animals treated with phenylalanine (Phe): (F) Jejunal loop; (G) Ileal loop; and (H) Colonic loop. (I) Exogenous IAP (10 U) enhances *E. coli* growth in the jejunal loop. (J) *E. coli* growth is reduced in the intestinal luminal fluid of IAP-KO mice compared to wild-type (WT) mice. Bacterial growth in the luminal fluid from the proximal small intestine (PSI), distal small intestine (DSI) and colon of IAP-KO mice compared to wild-type (WT) mice: (K) Growth of *E. coli* was significantly reduced in DSI luminal fluid of IAP-KO mice compared to wild-type (WT) mice; (L) Growth of *S. Typhimurium*. Groups of mice were euthanized and the luminal fluid from different segments or the entire intestine of each mouse was collected. Approximately, 1,000 colony-forming units (CFU) of bacteria were added to the fluid and incubated for 2 h at 37°C. Values are expressed as mean +/- SEM. Statistics: Statistical significance between two groups was tested using the two-tailed Student’s *t* test.; *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 2: ATP inhibits the growth of stool aerobic bacteria in vitro. Mouse stool samples were collected fresh directly in BHI media, homogenized, serially diluted and then cultured under aerobic conditions. For aerobic growth, each bacterial culture (200 μl) was grown in 8 wells of a 96-well clear-bottom plate at 37°C in a shaking incubator, and absorbance (OD₆₀₀) was determined at indicated time-points (see Materials and Methods). (A) Effects of different targets of IAP on stool aerobes. (B) The effect of ATP on bacterial growth was most evident when ODs were compared after 10 hours of incubation (one-way ANOVA F(4, 38) = 4.49, P value < 0.01; Tukey’s post hoc test: P value < 0.05). (C) Dose response effects of ATP on stool aerobes. (D) The highest effect of low concentration of ATP on bacterial growth was evident after 8 hours of incubation (one-way ANOVA: F(9, 68) = 49.30, P value < 0.001; Tukey’s post hoc test: P value = 0.015 for 200 μM of ATP, P values < 0.001 for 400, 800, 1600 μM of ATP). (E) Effects of ATP derivatives on stool aerobes. (F) These effects are also illustrated at the final time point. (G) IAP reverses the inhibitory effects of ATP on stool aerobic bacterial growth. (H) IAP abolished the effects of ATP and dose-dependently increased bacterial growth (one-way ANOVA: F(5, 83) = 13.97, P value < 0.001; Tukey’s post hoc test: P values < 0.001). Values for bacterial growth are expressed as average absorbance (OD₆₀₀) +/- SD. The experiment was repeated at least 3 times showing similar results.
**Figure 3:** ATP inhibits the growth of stool anaerobic bacteria *in vitro*. Mouse stool samples were collected fresh directly in BHI media, homogenized, serially diluted and then cultured under anaerobic conditions. For the growth of stool anaerobes, cultures were grown in a sealed plastic bag containing CO₂ producing gas pack and anaerobic condition indicators for 4 h at 37°C. Then the cultures were plated on Brucella (5% horse blood)-agar plates and incubated in a sealed plastic bag containing CO₂ producing gas pack and anaerobic condition indicators for 3 days at 37°C (see Materials and Methods). (A) Effects of different targets of IAP on the growth of stool anaerobes. (B) Dose response effects of ATP on the growth of stool anaerobes. (C) Effects of ATP derivatives on the growth of stool anaerobes. (D) IAP reverses the inhibitory effects of ATP on the growth of stool anaerobes. Values are expressed as mean CFU +/- SEM. Statistics: Two-tailed Student’s *t*-test; **p < 0.01, ***p < 0.001. The experiment was repeated at least 3 times showing similar results.
**Figure 4**: IAP reverses the bacterial growth-inhibition by various nucleotide triphosphates. Mouse stool samples were collected and grown under aerobic and anaerobic conditions as described in Figures 2 and 3 (also see Materials and Methods). Cultures were treated with a nucleotide (10 mM) +/- IAP (200 U/ml). Growth of stool aerobes in different nucleotide triphosphates: (A) ATP; (B) GTP; (C) CTP; (D) TTP; (E) UTP; (F) dATP; (G) dGTP; (H) dCTP and (I) dTTP. Values for aerobic bacterial growth are expressed as average absorbance (OD$_{600}$) +/- SD. The experiment was repeated at least 3 times showing similar results. (Two way repeated measures ANOVA: effect of time: F$_{(3, 19)}$ = 1330.55, P value < 0.001; interaction of time and IAP: F$_{(3, 19)}$ = 449.87, P value < 0.001; interaction of time and nucleotide triphosphate: F$_{(27, 63)}$ = 2.32, P value < 0.01; interaction of time, IAP and nucleotide triphosphate: F$_{(24, 63)}$ = 2.70, P value < 0.01; between groups differences for IAP: F$_{(1, 21)}$ = 516.65, P value < 0.001; between groups differences for nucleotide triphosphates: F$_{(9, 21)}$ = 11.61, P value < 0.001; Tukey’s post hoc test: P value < 0.05 for dTTP; P values < 0.001 for all other nucleotide triphosphates.) (J) Dose response curve at the end point of the experiment (10 h) (two way ANOVA: effect of IAP: F$_{(1, 39)}$ = 586.13, P value < 0.001; Effect of nucleotide triphosphate: F$_{(9, 39)}$ = 13.65, P value < 0.001; interaction of IAP and nucleotide triphosphate: F$_{(9, 39)}$ = 8.44, P value < 0.001; Tukey’s post hoc test: P value < 0.05 for dTTP; P values < 0.001 for all other nucleotide triphosphates; Tukey’s post hoc test: P value < 0.01 for GTP; P values < 0.001 for all other nucleotide triphosphates). (K) Growth of stool anaerobes in different nucleotides. Values are expressed as mean CFU +/- SEM. The experiment was repeated at least 3 times showing similar results.
**Figure 5**: ATP differentially inhibits the growth of specific bacteria. Each bacterial culture (200 μl) was grown in 8 wells of a 96-well clear-bottom plate at 37°C in a shaking incubator, and absorbance (OD₆₀₀) was determined at indicated time-points. Dose-response effects of ATP (1-20 mM): (A) *E. coli*, (B) *S. Typhimurium*, (C) *S. aureus*, and (D) *L. monocytogenes*. Effects of ATP (10 mM) +/- IAP (200 U/ml) on the growth of: (E) *E. coli* (p = 0.029), (F) *S. Typhimurium* (p = 0.18), (G) *S. aureus* (p = 0.003), and (H) *L. monocytogenes* (p = 0.007). (I-L) These effects are also illustrated at the final time points. Values for bacterial growth are expressed as average absorbance (OD₆₀₀) +/- SD. The experiment was repeated at least 3 times showing similar results. Statistics: The statistical analysis was performed comparing the OD values in the IAP vs. Vehicle group using two-tailed paired Student’s *t*-test. Note: To avoid cluttering the error bars are not shown in Figure 5A-D.
Figure 6: Nucleotide triphosphates preferentially inhibit Gram-positive bacteria. Each bacterial culture (200 μl) was grown in 8 wells of a 96-well clear-bottom plate at 37°C in a shaking incubator, and absorbance (OD$_{600}$) was determined at indicated time-points. Each culture was treated with an individual nucleotide (10 mM) +/- IAP (200 U/ml). Growth was recorded every 2 hours and the data presented here are from the 6-h time point. Values for bacterial growth are expressed as average absorbance (OD$_{600}$) +/- SD. The experiment was repeated at least 3 times showing similar results.
**Figure 7:** ATP inhibits bacterial growth *in vivo*. The small intestine was dissected out and luminal fluid was collected by gentle squeezing, then centrifuged, and the supernatant was obtained for ATP assay (see Materials and Methods). For determining ATP concentration and studying the effects of ATP *in vivo*, laparotomy was performed on mice (*n* = 5 per group) under general anesthesia and a 5-cm jejunal loop was constructed. Approximately 1,000 CFU of a specific bacterial species were instilled by injection into the loop. After 2 h, the loop was dissected out, homogenized and plated on selective media for overnight growth at 37°C (see Materials and Methods for details). (A) ATP concentrations in the small intestinal luminal fluids of WT and IAP-KO mice. (B) ATP concentrations in the small intestinal luminal fluids of WT mice fasted for 14 h. (C) ATP concentrations in the jejunal loops of WT and IAP-KO mice. (D) ATP concentrations in the jejunal loops of IAP-KO mice receiving IAP (100 U) injection directly into the loop. (E) Growth of *E. coli* in the jejunal loops of WT mice pre-treated with 10 mM phenylalanine (Phe) in the drinking water. (F) Growth of *E. coli* in the jejunal loops of WT mice receiving the ecto-ATPase inhibitor ARL 67156 (10 mM) injection directly into the loop. Values are expressed as mean +/- SEM. Statistics: Two-tailed Student’s *t*-test; *p* < 0.05, **p** < 0.01, ***p** < 0.001.
Figure 8: Gut microbiota live in symbiosis with the host. The brush border enzyme IAP appears to play a central role in regulating the microbiota through a mechanism that involves the dephosphorylation of luminal ATP. ATP (and similarly other nucleotide triphosphates not shown in this figure) exerts an inhibitory effect on the growth and survival of a wide spectrum of bacteria. By dephosphorylating ATP, IAP blocks this inhibitory effect, resulting in greater numbers of gut bacteria. Both aerobes and anaerobes are affected by ATP. The selected gram positive bacteria (*L. monocytogenes*, *S. Aureus*) were more significantly affected by ATP and consequently IAP as compared to the tested gram negative (*E. Coli*) and the pathogen tested (*Salmonella*) was not significantly affected by ATP/IAP.
Figure 1

A. Jejunal Loop

E. coli growth (fold)

B. Jejunal Loop

M. morganii growth (fold)

C. Jejunal Loop

E. faecalis growth (fold)

D. WT : Jejunal Loop

E. coli growth (fold)

E. Jejunal Loop

IAP levels (U/ml luminal fluid)

F. WT : Jejunal Loop

E. coli growth (fold)

G. WT : Ileal Loop

E. coli growth (fold)

H. WT : Colonic Loop

E. coli growth (fold)

I. KO : Jejunal Loop

E. coli growth (fold)

J. Luminal Fluid

E. coli growth (fold)

K. Luminal fluid

Growth of E. coli (CFU)

L. Luminal fluid

Growth of S. Typhimurium (CFU)
Figure 2

A) Growth of Stool Aerobes (OD_{600})

- LPS (10 µg/ml)
- CpG (10 µg/ml)
- Flagellin (100 ng/ml)
- ATP (10 mM)
- Control (no substrate)

B) ATP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control
- ATP (10 mM)
- ADP (10 mM)
- AMP (10 mM)
- Phosphate (10 mM)

C) ATP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no ATP)
- ATP (25 µM)
- ATP (50 µM)
- ATP (100 µM)
- ATP (200 µM)
- ATP (400 µM)
- ATP (800 µM)
- ATP (1,600 µM)

D) ATP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no ATP)
- ATP (25 µM)
- ATP (50 µM)
- ATP (100 µM)
- ATP (200 µM)
- ATP (400 µM)
- ATP (800 µM)
- ATP (1,600 µM)

E) ATP + IAP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no substrate)
- ATP + IAP (5 U/ml)
- ATP + IAP (10 U/ml)
- ATP + IAP (20 U/ml)
- ATP + Vehicle

F) ATP + IAP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no ATP)
- ATP (10 mM)
- ADP (10 mM)
- AMP (10 mM)
- Phosphate (10 mM)

G) ATP + IAP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no substrate)
- ATP + IAP (5 U/ml)
- ATP + IAP (10 U/ml)
- ATP + IAP (20 U/ml)
- ATP + Vehicle

H) ATP + IAP Concentration vs. Growth of Stool Aerobes (OD_{600})

- Control (no ATP)
- ATP (10 mM)
- ATP + IAP (5 U/ml)
- ATP + IAP (10 U/ml)
- ATP + IAP (20 U/ml)
- ATP + Vehicle
Figure 3

A

Growth of stool anaerobes (CFU)

Incubation (h) 0 4 4 4 4 4
IAP substrate — ATP LPS CpG Flagellin

B

Growth of stool anaerobes (CFU)

Incubation (h) 0 4 4 4 4 4
ATP (µM) — 25 50 100 200

C

Growth of stool anaerobes (CFU)

Incubation (h) 0 4 4 4 4 4
IAP substrate — ATP ADP AMP Phosphate

D

Growth of stool aerobes (OD)

ATP (mM) — 5 5 5 5 5 5
IAP (U/ml) — 1.5 3.1 6.2 12.5 25 50
Figure 4

A. Growth of stool aerobes after 10h (OD600) with ATP (10 mM) + IAP (200 U/ml) compared to ATP (10mM), Control.

B. Growth of stool aerobes after 10h (OD600) with GTP (10 mM) + IAP (200 U/ml) compared to GTP (10mM), Control.

C. Growth of stool aerobes after 10h (OD600) with CTP (10 mM) + IAP (200 U/ml) compared to CTP (10mM), Control.

D. Growth of stool aerobes after 10h (OD600) with TTP (10 mM) + IAP (200 U/ml) compared to TTP (10mM), Control.

E. Growth of stool aerobes after 10h (OD600) with UTP (10 mM) + IAP (200 U/ml) compared to UTP (10mM), Control.

F. Growth of stool aerobes after 10h (OD600) with dATP (10 mM) + IAP (200 U/ml) compared to dATP (10mM), Control.

G. Growth of stool aerobes after 10h (OD600) with dGTP (10 mM) + IAP (200 U/ml) compared to dGTP (10mM), Control.

H. Growth of stool aerobes after 10h (OD600) with dCTP (10 mM) + IAP (200 U/ml) compared to dCTP (10mM), Control.

I. Growth of stool aerobes after 10h (OD600) with dTTP (10 mM) + IAP (200 U/ml) compared to dTTP (10mM), Control.

J. Growth of stool aerobes after 10h (OD600) for different nucleotides (ATP, GTP, TTP, CTP, UTP, dATP, dGTP, dTTP, dCTP) with Control, IAP–, IAP+.

K. Growth of stool anaerobes (CFU) for different nucleotides (ATP, GTP, TTP, CTP, UTP, dATP, dGTP, dTTP, dCTP) with Control, IAP–, IAP+. 

Nucleotides 10 mM
**Figure 5**

A. 

E. coli growth (OD600) over time with different ATP concentrations.

B. 

S. Typhimurium growth (OD600) over time with different ATP concentrations.

C. 

S. aureus growth (OD600) over time with different ATP concentrations.

D. 

L. monocytogenes growth (OD600) over time with different ATP concentrations.

E. 

Comparison of growth of different bacteria with ATP (10 mM) and IAP (200 U/ml).

F. 

ATP (10 mM) + IAP (200 U/ml) effect on E. coli growth.

G. 

ATP (10 mM) effect on S. aureus growth.

H. 

ATP (10 mM) effect on L. monocytogenes growth.

I. 

ATP (10 mM) effect on S. Typhimurium growth.

J. 

Comparison of S. Typhimurium growth between ATP (10 mM) and IAP (200 U/ml).

K. 

Comparison of S. aureus growth between ATP (10 mM) and IAP (200 U/ml).

L. 

Comparison of L. monocytogenes growth between ATP (10 mM) and IAP (200 U/ml).
Figure 6

A

Growth of E. Coli (OD600)

Control
IAP^-
IAP^+

ATP  GTP  TTP  CTP  UTP  dATP  dGTP  dTTP  dCTP

B

Growth of S. Typhimurium (OD600)

Control
IAP^-
IAP^+

ATP  GTP  TTP  CTP  UTP  dATP  dGTP  dTTP  dCTP

C

Growth of S. Aureus (OD600)

Control
IAP^-
IAP^+

ATP  GTP  TTP  CTP  UTP  dATP  dGTP  dTTP  dCTP

D

Growth of L. Monocytogenes (OD600)

Control
IAP^-
IAP^+

ATP  GTP  TTP  CTP  UTP  dATP  dGTP  dTTP  dCTP
Figure 7

A: Small Intestinal Luminal Fluid

B: Small Intestinal Luminal Fluid

C: Jejunal Loop

D: KO: Jejunal Loop

E: WT + Phe: Jejunal Loop

F: WT: Jejunal Loop

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