KRESSER INSTITUTE FOR FUNCTIONAL AND EVOLUTIONARY MEDICINE

Gut Diagnosis: Stool Testing Review

The two primary categories of techniques for stool testing are:

1. Culture-based techniques:
    - Includes high-complexity culture and proteomic-based spectrometry (matrix-assisted laser desorption/ionization time-of-flight [MALDI-TOF]) stool testing.
    - Proteomics entails the identification and characterization of proteins in cells, tissues, or organisms. These signature proteins (protein fingerprints) are matched against a database that enables identification of over 1,400 microbial species of bacteria, fungi, and yeast very quickly.
    - The MALDI-TOF method has been shown to have 99 percent accuracy in the identification of common commensal microbes, 100 percent accuracy in the identification of common pathogenic species, and high reproducibility across laboratories.
    - One limitation of culture-based methods like MALDI-TOF is that they rely on successfully culturing the microbe first. Because less than 5 percent of microbes in the human gut are typically picked up using commercial laboratory cultures, the MALDI-TOF method does not tell us about abundance.
2. Molecular or sequence-based techniques:
    - **DNA-polymerase chain reaction (PCR)** - very accurate for identifying microorganisms but requires a specific primer for each microorganism, which increases the cost for the lab. DNA-PCR may be able to provide susceptibility information, but the use of this is still clinically limited.
    - **16s rRNA gene sequencing** - identifies bacteria at a genus level, without species or strain information. There's no eukaryote, fungi, or parasite testing, so it's not comprehensive. It is also subject to primer bias, which can reduce the accuracy of the relative abundance of each microbe in the sample. Popular companies offering this type of sequencing include Thryve, American Gut Project, and Atlas Biomed.
    - **Whole-genome sequencing** - likely to provide more information on microbial richness and diversity, but the clinical utility at this stage is still lacking and it needs to be combined with other stool testing techniques to provide well-rounded results and data. Our current lab preference for this method is Onegevity because of their techniques and reproducibility. Eventually, this

method has the potential to provide affordable and clinically relevant information.

When the goal is to use the stool test diagnostically on an individual subject and apply that information to make clinical decisions, we feel the most appropriate methodology is PCR, and a quantitative method such as qPCR is preferred. See the following comparison chart from the *Townsend Letter*:

Stool Testing Methods	Fully quantitative*	Highly sensitive detection *(Measures very low levels of organisms)*	Each analyte individually validated	Provides only Clinically Relevant Organisms	Rapid turn around time *(within days)*	Identifies bacteria, parasites, fungi, and viruses down to the strain level	Identifies genes involved in microbial function
qPCR / rt-PCR	+++	+++	+++	++	+++	+++	++
Standard PCR	–	++	++	++	+++	++	++
Shotgun Metagenomic Sequencing	–	+	–	–	–	++	+++
Metatranscriptomic Sequencing	–	+	–	–	–	++	+++
16S Sequencing	–	+	–	–	–	–	–
Culture + MALDI-TOF MS	–	–	+	+	+	–	–
Microscopy	–	–	+	+	+	–	–

Adapted from: https://www.townsendletter.com/article/450-diagnostic-stool-testing-methodology/

Doctor's Data

GI360 Profile:

- Uses multiplex PCR technology, coupled with the MALDI-TOF proteomics.
- Identifies and characterizes abundance and diversity of more than 45 analyses and identifies the presence of pathogenic viruses, bacteria, and parasites.
- Provides a dysbiosis index based on the patient's overall bacterial abundance and profile, as compared to a reference population.
- Provides biomarkers for digestion, absorption, inflammation, and immune status, as well as short-chain fatty acid production, stool pH, white blood cells, red blood cells, mucus, occult blood, and more.

Genova Diagnostics

GI Effects Comprehensive Stool Profile:

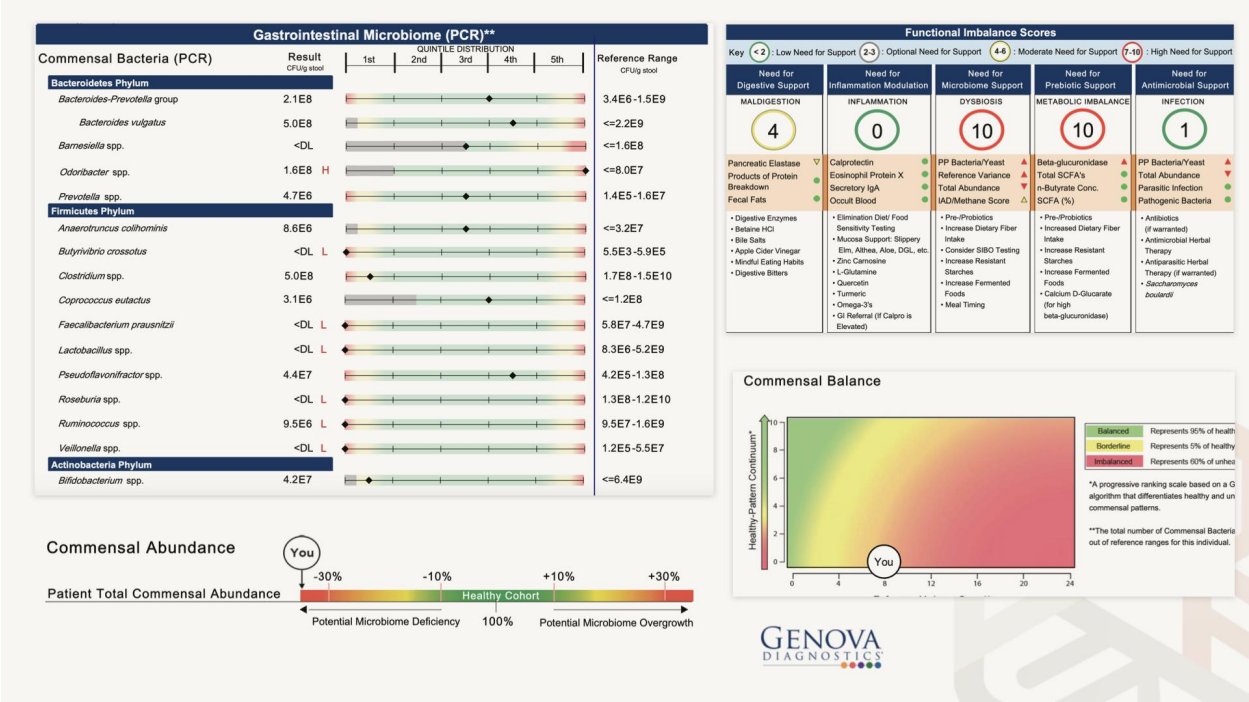
- Uses a combination of PCR, culture, and microscopic methods.
- Evaluates the composition of gut flora and identifies the presence of pathogenic viruses, bacteria, and parasites using culture and PCR testing.
- Provides biomarkers for digestion (pancreatic elastase, fecal fat) and inflammation and immunology (calprotectin, fecal secretory immunoglobulin A [SIgA]).
- Recovers live organisms like yeast and bacteria for susceptibility testing and add-on biomarkers that can include *Campylobacter*, *Helicobacter pylori*, lactoferrin, zonulin, and a few more.

Diagnostic Solutions Laboratory

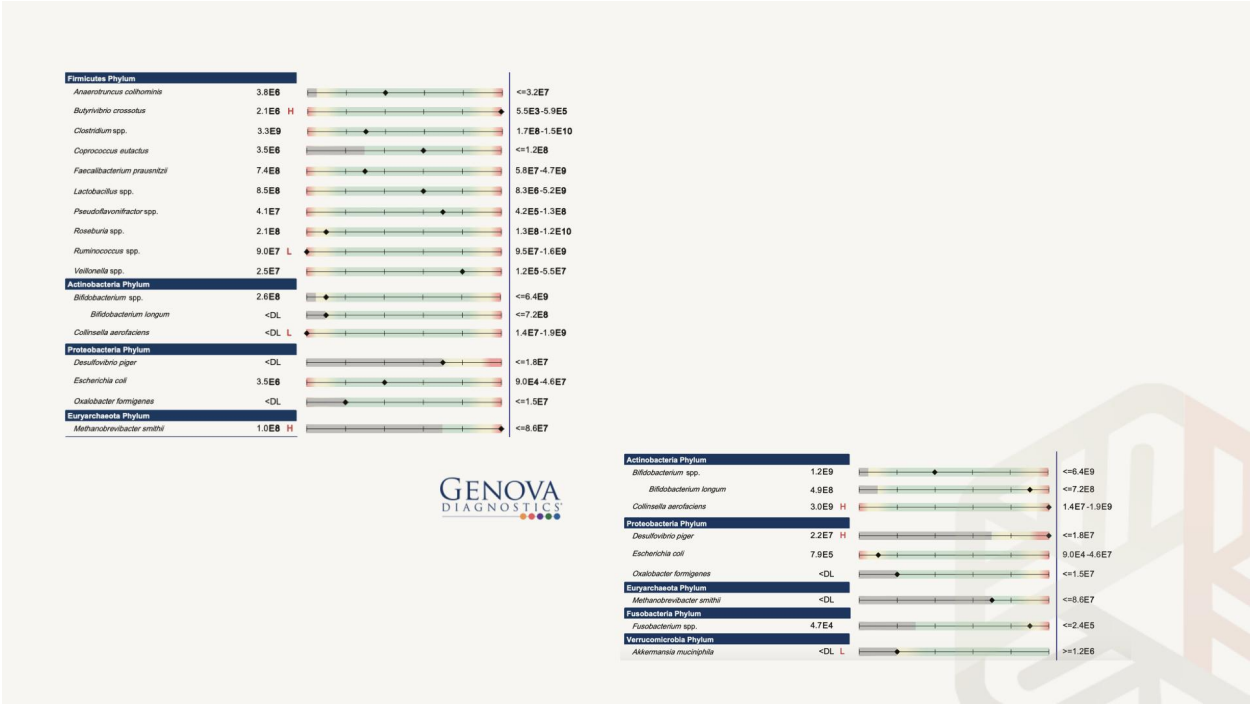
GI-MAP | GI Microbial Assay Plus

- Relies exclusively on qPCR, so it requires a bit more clinical correlation with symptoms and toxin testing to determine appropriate treatment since the presence of DNA material or pathogenic strain does not always indicate illness.
- Detects commensal flora as well as bacterial, parasitic, and viral pathogens.
- Provides biomarkers for intestinal health and tests for *H. pylori*, including virulence factors.
- Measures antibiotic resistance using antibiotic-resistant genes rather than the gold standard of culture and sensitivity.

Beneficial Bacteria



In this Genova Diagnostics report, commensal balance and abundance are reported in several ways. We can see it's a case of dysbiosis that is in part insufficiency of beneficial bacteria and also pretty significant overgrowth of a couple of pathogenic species.



The groups worth mentioning in the beneficial bacteria section are the methanogens and the sulfate-reducing organisms.

- The most dominant methanogen and probably the most represented on stool testing is the *Methanobrevibacter*. While there's no real research or support literature for using methanogens in stool to diagnose methane-dominant small intestinal bacterial overgrowth (SIBO), higher levels tend to be seen in patients with irritable bowel syndrome (IBS) with constipation.
- The sulfate-reducing organisms include *Desulfovibrio* species, *Fusobacterium*, and sometimes *Escherichia coli*. The most prevalent and dominant of those is the *Desulfovibrio* species. It is suspected that higher quantities seen in the stool represent more potential for hydrogen sulfide production and a higher capacity for sulfate metabolism. As a trend, these higher density numbers tend to be seen more often in patients with IBS with diarrhea.

Potential Pathogens

Klebsiella:

- It can be a normal resident of the digestive tract but can become overgrown in dysbiosis.

- It is associated with joint pain and autoimmune conditions like ankylosing spondylitis, reactive arthritis, and rheumatoid arthritis.
- It is also reported in IBS and other gut issues.
- When you see a positive result for *Klebsiella*, especially in someone with joint pain, you should run the HLA-B27 test.
- Patients with ankylosing spondylitis have elevated levels of antibodies to *Klebsiella*. The theory is this is due to molecular mimicry.
- Only a small percentage of people with the HLA-B27 gene develop ankylosing spondylitis, which suggests there may be an environmental trigger.
- Some of the literature is now looking at the effectiveness of a low-starch diet for people who have *Klebsiella* and HLA-B27.
- You may want to use antimicrobials and recommend a low-starch diet for these patients.

Citrobacter Freundii

- A facultative aerobic gram-negative bacilli of the *Enterobacter* family and close relative of the better-known food poisoner, *Salmonella*.
- Often found in soil, water, sewage, food, and intestinal tracts of animals and humans. It's also known to be the cause of a number of nosocomial infections of the respiratory tract, urinary tract, blood, and many other normally sterile sites in patients.
- Represents about 29 percent of all opportunistic infections; some subspecies are more pathogenic than others.

Helicobacter pylori:

- Associated with stomach and duodenal ulcers
- Possibly increases the risk of gastric cancer

H. pylori: pathogen or not?

1. Dr. Martin Blaser gathered evidence suggesting that *H. pylori* is not always harmful. It may even be helpful in some circumstances. Dr. Blaser's research shows that *H. pylori* has beneficial functions that actually begin in infancy if a baby acquires it. For example, it appears to protect against the development of allergies and asthma.
2. In animal studies, if the animals are infected early in life, such as shortly after birth or during infancy, *H. pylori* has been shown to play a protective role.
3. Another factor determining the pathogenicity of *H. pylori* is the particular strain. Some strains of *H. pylori* appear to be more pathogenic than others.

Treatment decision

Children	Young adults (<30)	Older adults (>30)
No evidence that eliminating *H. pylori* results in benefits	Evidence is unclear	Benefits may outweigh potential harm, but full eradication may not be necessary
Some evidence that eradication may cause harm	May be benefit, but may also be harm (increased risk of obesity, allergies, or asthma)	Eradication of *H. pylori* may increase esophageal cancer, GERD, obesity

Doctor's Data GI360 and Genova GI Effects offer *H. pylori* stool antigen tests as add-ons. At the time of this recording, Diagnostic Solutions Laboratory is the only easily accessible stool test that's offering the virulence factors.

Another option to test for *H. pylori* is a breath test, and this is especially accurate for post-treatment follow-up testing, but it does require an entirely separate kit. A blood test for *H. pylori* is not recommended because it can't distinguish between previous exposure and current exposure.

Yeast and Fungi:

- These are normal residents of the digestive tract, but they can be a problem if they are overrepresented.
- Common causes of yeast overgrowth include antibiotic use, hypochlorhydria, impaired immune function, dysbiosis, and high intake of sugars and starches consistent with a Standard American Diet.
- Fungi secrete toxins that can damage the intestinal lining and provoke inflammation. They compete for adhesion sites with beneficial bacteria. They are hyphaic and can puncture the gut lining and make it permeable.
- Some species of *Candida* secrete substances that have SIgA protease activity. This means that the *Candida* can secrete toxins that can break down SIgA.
- You'll often see low SIgA in patients with fungal overgrowth.

In Doctor's Data GI360, yeast is evaluated via two methods:

1. Yeast culture
    a. Can get a false negative as yeast has a patchy distribution in stool.
    b. If culture is negative, consider the patient's symptoms and microscopy results.
2. Microscopic examination
    a. "None," "rare," or "few" is the expected finding for microscopic yeast.
    b. "Moderate" or "many" indicates fungal overgrowth.

pH:

There is a strong correlation between an **acidic environment** and **fungal overgrowth**. Too much alkaline can create an environment that is conducive to overgrowth of harmful bacteria. pH is largely dependent on short-chain fatty acid (SCFA) production.

PARASITES:

- Detection is difficult in stool; therefore, you often need to use more than one sample.

Blastocystis:

- Most common parasite in North America.
    - May be a normal resident of the gut. DNA/PCR studies have found *Blastocystis* in 80 percent of healthy individuals.

Several factors determine whether it will be benign or pathogenic in a given individual.

- Likely nonpathogenic in healthy hosts
    - Our gut is an ecological system. If it's healthy, it's resistant to pathogens, and if it's not, it will be susceptible to pathogens.
- Pathogenic in people with underlying health conditions.
- Multiple types of *Blastocystis*, some being more pathogenic.
    - At the time of this recording, the only commercial lab offering subtyping as a part of their stool testing is Genova Diagnostics, in their GI Effects Comprehensive stool panel, and some other panels that include parasite testing.
- Treatment is not risk-free, so the decision to treat requires clinical judgment.

For example, if *Blastocystis* was positive in all three stool samples on a patient who felt great, had no symptoms, hadn't taken any antibiotics for 20 years, and was as healthy as could be, I would definitely not recommend treatment.

However, if *Blastocystis* comes back positive on a patient with a long history of digestive problems, fatigue, eczema, acne, low libido, insomnia, and other symptoms, and if they are positive for other parasites, then an antiparasitic protocol might be warranted.

Blastocystis symptoms:

Wide variety of GI issues	**Extra-intestinal symptoms**
Pain, gas, bloating, diarrhea, constipation, greasy stools that tend to float, nausea.	Fatigue, skin rash, brain fog, joint pain.

Dientamoeba fragilis

- *It is* very often a co-infection with pinworm.
- It is unclear if commensal or pathogenic.
- Symptoms are similar to *Blastocystis*.
- If you find *D. fragilis*, consider further testing/treating for pinworm because that's how it's often transmitted.
- **BadBugs.org** is a website that's a great source of information for *Blasto* and *D. fragilis*.

Endolimax nana

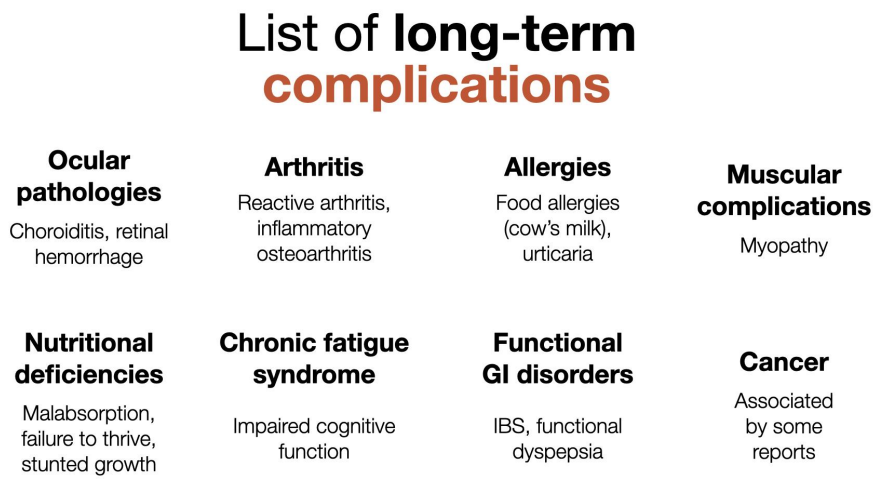
- Nonpathogenic in immunocompetent people.
- Pathogenic in immunocompromised people.
- Common co-infection with *Blastocystis*.
- If you see it on a test alone, there is concern of missing others; consider retesting. (Do a follow-up test with a specialized lab such as **Parasitology Center**.)

Entamoeba coli

- *It is* generally considered to be a nonpathogenic commensal parasite.
- There are two concerns:
    - Often occurs with other pathogenic parasites.
    - Sometimes confused with *Entamoeba histolytica* (highly pathogenic).
- If you see it on a test alone, consider retesting, as there is concern of missing others.

Giardia

- Immunoassays or antigen detection are preferred due to difficulty detecting in stool.
- Universally considered pathogenic, so treat if detected.
- Many associated complications.



These complications may persist even after eradication.

Cryptosporidium

- Similar symptoms to other parasites, with watery diarrhea most common in the acute phase.
- Can be serious to life-threatening in immunocompromised patients.
- Often self-limiting, but reinfection and chronic infection are possible.
- Rare to find on Doctor's Data.
- Direct fluorescent antibody technique is 99 percent sensitive and almost 100 percent specific. PCR testing is now the primary methodology for parasite testing by Doctor's Data, Genova Diagnostics, and Diagnostic Solutions Laboratory.
- Treat if detected.

Charcot-Leyden crystals

- Evidence of eosinophil breakdown. Therefore, often seen in parasitic disease.
- If you see them but no parasite, consider a retest with a different lab to make sure a parasite was not missed.

OTHER MARKERS:

Red blood cells in stool

- Associated with parasitic or bacterial infections.
- Also associated with inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis.
- Check for invasive gut pathogens like *Shigella*, *Campylobacter*, and *Yersinia*, which can cause mucosal inflammation and bleeding.

Fecal elastase

- Pancreatic enzyme that digests and degrades proteins.
- Low indicates pancreatic exocrine insufficiency.
    - Moderate: 100 to 200
    - Severe: <100
- Highly specific for small intestinal disease.
- Pancreatic insufficiency may be a risk factor for SIBO, especially recurring SIBO.

Fat stain

- Indicator of fat malabsorption, which is often secondary to pancreatic or biliary tract disease.
- If you see a positive fat stain, consider gastric surgery, pancreatic disease, biliary obstruction, liver disease, or intestinal permeability.
- Supplementation with pancreatic enzymes, hydrochloric acid (HCl), and/or bile may be helpful.

Muscle fibers

- Marker of incomplete protein digestion.
- May also be seen with inadequate chewing.
- Can suggest hypochlorhydria or insufficient enzyme production. Check if the patient is on Proton-pump inhibitors or acid-suppressing drugs.
- Digestive enzymes or HCl can be helpful.

Vegetable fibers

- Indicates carbohydrate malabsorption, which can be a risk factor or effect of SIBO.
- Digestive enzymes or HCl can be helpful.

INFLAMMATORY MARKERS:

Lactoferrin

- Protein in the transferrin family that's expressed in activated neutrophils.
- Significantly elevated lactoferrin is a marker for IBD.
- Marker for gut inflammation/infection at lower levels.
- Many labs offer it as an add-on to the stool test, so if you know or suspect that your patient has IBD, make sure lactoferrin is included.

Calprotectin

- Marker of GI inflammation in the mucosa and presence of neutrophils.
- May be more accurate for IBD diagnosis than lactoferrin.

>200 µg/g	50-200 µg/g	<50 µg/g
Active IBD, colitis, cancer	Chronic inflammation, NSAIDs, inactive IBD	Normal

- If calprotectin is >200 or lactoferrin is >50, run an IBD expanded antibody panel with Labcorp (test #162045). Example report interpretation below:

Positive	Negative	Interpretative Report
Only **pANCA**	-	*"Suggestive of ulcerative colitis."*
Only **one** of **ACCA, ALCA, AMCA** or **gASCA**	***pANCA***	*"Suggestive of Crohn's disease. Pattern is not conclusive for disease behavior risk stratification."*
Only **one** of **ACCA, ALCA, AMCA** or **gASCA** + ***pANCA***	-	*"Suggestive of inflammatory bowel disease. Pattern is not conclusive for any specific disease form."*
Any **two** of **ACCA, ALCA, AMCA** or **gASCA** + ***pANCA***	***pANCA***	*"Suggestive of Crohn's disease with high risk of aggressive disease behavior (development of strictures or fistulae)."*
Any **three** or more of **ACCA, ALCA, AMCA or gASCA** + ***pANCA***	***pANCA***	*"Suggestive of Crohn's disease with the very high risk of aggressive disease behavior (development of strictures or fistulae)."*
-	**ALL markers**	*"Pattern is not suggestive of inflammatory bowel disease."*

- If any three or more of the antibodies are positive, that's also suggestive of Crohn's disease, with a very high risk of aggressive disease behavior.

Lysozyme

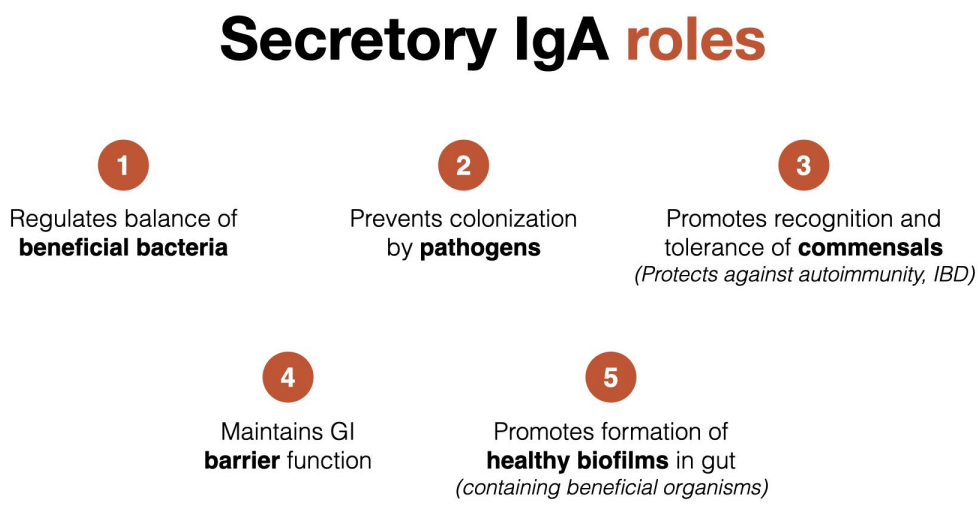
- Lysozyme is an enzyme that catalyzes the hydrolysis of specific glycosidic bonds in mucopolysaccharides that constitute the cell wall of Gram-positive bacteria.
- General marker for gut inflammation.
- Moderately elevated level associated with overgrowth or food antigens.
- Can be treated with anti-inflammatories or removing the offending agent.
- High levels associated with IBD and non-IBD GI disease with diarrhea; often require further testing.

Fecal lysozyme
disease association

600-2,000 ng/mL	>2,000 ng/mL
Yeast, dysbiotic bacteria, parasites	Active IBD

Secretory IgA

- First line of defense against entry of enteric toxins and pathogenic organisms from the colon.
- Best used as a marker to determine outcomes of treatment.
- Can take several months to normalize.
- High levels may indicate activation of the gut immune system, possibly due to a viral or bacterial pathogen, or a more chronic, pathological issue.
- Low levels may indicate chronic problems and increase the risk of dysbiosis, pathogen invasion, and leaky gut. Fungal overgrowth is one possible cause.
- With extremely low or undetectable levels, consider ordering a quantitative immunoglobulin panel through Labcorp or Quest to rule out genetic deficiencies.



White blood cells and mucus

- Occur with bacterial and parasitic infections and gut inflammation.

Short-chain fatty acids

- Produced by beneficial bacteria in the gut.
- Are the end product of bacterial fermentation of fermentable carbohydrates.
- Decrease the pH to make an unsuitable environment for pathogens.
- Decrease inflammation and increase T-regulatory cell production/differentiation.
- Stimulate growth and repair of enterocytes, the cells lining the GI tract.