

Investigation of Serum Derived Bovine Immunoglobulin IgG Binding of Various Antigens by Modified ELISA



Summary

Serum-derived bovine immunoglobulin/protein isolate (SBI) is a plasma fraction rich with immunoglobulins formulated for oral administration. SBI reduces inflammation in the gastrointestinal tract by both immune and steric exclusion mechanisms resulting from oral immunoglobulins binding proinflammatory antigens. previously SBI has been shown to bind to common intestinal antigens which include LPS, Lipid A, and the ligand Pam3CSK4 using a modified ELISA. Employing the same modified ELISA setup, this study expanded the list of antigens to which SBI can bind and therefore the situations in which SBI may reduce inflammation. The IgGs contained in SBI bind *C. albicans* lysate and Als3 protein, *H. pylori* CagA protein, cytolethal distending toxin subunits A and C, and Shiga-like toxin 1 subunit. Specificity of IgG for the listed antigens was determined based upon the comparison of blocking, background, and denatured SBI controls to the experimental groups. Dot blot screening of *H. pylori* lysate and aflatoxin M1 showed no IgG-antigen binding. Lack of SBI IgG binding to *H. pylori* lysate is in conflict previously published data and maybe due to differences in strain. Absence of SBI binding to aflatoxin M1 maybe due to the focus on IgG binding as bovine serum albumin (BSA) is known to bind some mycotoxins and BSA comprises approximately 10% of SBI protein content.

Table 1. Antigens tested for SBI-IgG binding. Specific binding of IgG to antigens determined by ELISA. Negative results determined by dot blot.

Antigen	IgG Specifically Binds Antigen via ELISA
<i>C. albicans</i> lysate	Yes
<i>C. albicans</i> Als3 protein	Yes
<i>H. pylori</i> lysate	No
<i>H. pylori</i> CagA protein	Yes
Cytolethal distending toxin subunit A	Yes
Cytolethal distending toxin subunit C	Yes
Shiga-like toxin type 1	Yes
Aflatoxin M1	No

INTRODUCTION

The health and performance of the gut is influenced by a myriad of factors including diet, genetics, environment, stress, intestinal barrier, immune status, and the microbiome. The alteration of one or more of these factors can lead to enteropathy, which is characterized as an increase in intestinal permeability, impairment of gut immune function, and nutrient malabsorption¹. An imbalance in the gastrointestinal tract can lead to deteriorating intestinal barrier function allowing for the translocation of pathogens and induce circulating immune regulators. Thus, generating a cyclical cause and effect relationship of increased inflammation and aberrant cytokine production². The positive feedback loop of inflammation in the gut can ultimately cause chronic disease states such as IBS-D, IBD, or HIV-associated enteropathy. Therefore, restoring balance within the GI tract is critical to overall health.

Immunoglobulins have a positive role in gut homeostasis. Serum-derived bovine immunoglobulin/protein isolate (SBI) is a spray-dried protein powder specially formulated to enrich the immunoglobulin content to help manage loose and frequent stools associated with gastrointestinal enteropathies. It is manufactured by fractionating edible grade bovine plasma to contain 90% protein, with most of the total protein comprised of immunoglobulins: 60% IgG, 5% IgM, and 1% IgA. The high concentration of immunoglobulins has shown to bind and neutralize opportunistic pathogens by immune and steric exclusion mechanisms using an *in vitro* cell culture model³. This binding of antigens helps limit the stimulation of immune regulators that modulate chronic intestinal inflammation when these microbial components translocate into the lamina propria. The goal of this study was to expand the list of antigens to which SBI binds by adapting the ELISA used by Detzel *et al*³ to demonstrate binding of IgG to lipopolysaccharide, lipid A, and Pam3CSK4 antigens. New relevant antigens of interest were chosen based on the market needs for binding of gram-negative bacteria components and mold mycotoxins.

Overview of Antigens

Helicobacter pylori

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral shaped bacterium that colonizes the human gastric mucosa. It is estimated that over half the world's population carries *H. pylori*. Infection can lead to chronic atrophic gastritis, peptic ulcers, or gastric adenocarcinoma.

Clinically, cytotoxicity-associated immunodominant antigen (CagA) positive *H. pylori* strains are more pathogenic and lead to disease states. CagA is injected directly into gastric epithelial cells by *H. pylori* and negatively influences tight junctions, cell proliferation, and morphology⁴.

Candida albicans

Candida albicans (*C. albicans*) is a commensal yeast found commonly in the human oral mucosa, gut, vagina, and skin. When found in a dysbiosis state, *C. albicans* become pathogenic causing superficial and systemic infections. *C. albicans* are dimorphic and highly adaptable to change in environment (pH, temperature, CO₂, nutrients).

The agglutinin-like protein 3 (Als3) plays a diverse role in *C. albicans* infection. Als3 functions as an adhesin, mediating attachment to epithelial cells, endothelial cells, and matrix proteins. It also has an active role in

biofilm production and helps with iron uptake⁵. Lastly, Als3 is an invasin, binding to host cells receptors like E- and N-cadherin to endocytose the host cell⁶.

Cytolethal distending toxin

Cytolethal distending toxins (CDT) are heterotrimeric toxins produced by gram-negative bacteria associated with gut inflammation and disease. CDT activity relies on three subunits: CdtA, CdtB, and CdtC. CdtA and CdtC bind to the cell surface while CdtB acts as a nuclease, damaging host cell DNA resulting in cell cycle arrest. The individual domains display little to no toxicity until associating into a trimer of two cell binding domains and one CdtB. Cells infected with CDT display cytoplasm distention toxicity which gives the toxin its name⁷.

Shiga Toxin

Shiga toxin (Stx) is produced by both *Shigella dysenteriae* and some serogroups of *Escherichia coli*. Stx consist of two major subunits, A and B, with a single A subunit noncovalently binding to a pentamer of five identical B subunits. The subunits of the B pentamer bind to the cellular receptor, globotriaosylceramide, Gb3, found primarily on endothelial cells. Whereas the A subunit blocks protein synthesis by removing an adenine residue from the 28S rRNA of the 60S ribosome. Infection of Stx causes bloody diarrhea, abdominal pain, and is associated with Hemolytic Uremic Syndrome (HUS). Shiga toxin types 1 is produced by *E. coli* but is virtually identical to Stx produced by *S. dysenteriae*, containing only a single amino acid difference in primary sequence^{8,9}.

Mycotoxins

Mycotoxins are secondary toxic metabolites produced by certain fungi. Aflatoxin M1 (AFM1) is produced by *Aspergillus flavus* and can be found in milk and other food products obtained from contaminated livestock. AFM1 is a metabolite of aflatoxin B1 (AFB1) which is found in maize and nuts and is known to cause liver cancer. AFM1 is possibly carcinogenic and is regulated by the US Food and Drug administration¹⁰.

METHODS

Sample Preparation

A 10% SBI solution was prepared by dissolving 5 g of protein in 45 mL of deionized (DI) water. The solution was mixed for a minimum of 2 hours and centrifuged. Solutions were then filtered through a 0.45 µm syringe filter (Thermo Scientific, Waltham, MA). To prepare the denatured SBI control, a 10% solution was heated to 90°C, then allowed to cool. Denaturation was verified by low recovery of active IgG content by a turbidimetric immunoassay used for release testing ImmunoLin® product. The denatured protein control was then analyzed by dot blot to verify the ability of the detection antibody (sheep anti-bovine-HRP) to detect denatured IgG.

Materials

H. pylori lysate (MBS537291), CagA (MBS596143), agglutinin-like protein 3 (MBS1111464), Shiga like toxin-1 subunit B (MBS145496), cytolethal distending toxin subunit C (MBS1466408), and cytolethal distending toxin subunit A (MBS1308926) were purchased from MyBiosource, San Diego, CA. *Candida albicans* lysate (NAT41601-100) was purchased through CedarLane Labs, Burlington, NC. Aflatoxin M1-BSA (A6412) and

carbonate-bicarbonate (C3401-100CAP) were purchased from Sigma, St. Louis, MO. Tris (T393), sodium chloride (S640), methanol (A412), sulfuric acid (SA200), and Corning clear polystyrene 96-well medium-binding microplates (07-200-98) were purchased from Fisher Scientific, Hampton, NH. PVDF transfer membrane (88518), Tween 20 (PI37538), Pierce Protein-Free (PBS) Blocking Buffer (37572), 3,3', 5, 5'-tetramethylbenzidine (TMB) (N301), and SuperSignal Pico Chemiluminescent Substrate (34078) were purchased from Thermo Fisher Scientific, Waltham, MA. Sheep anti-bovine IgG-HRP (A10-118P) was purchased from Bethyl Laboratories, Montgomery, TX.

SBI–Antigen Binding: Dot Blot

A PVDF transfer membrane was activated in methanol for 30 seconds, washed, and transferred to water to equilibrate for 2 minutes. The membrane was then dotted with 5 μ L of antigen (200, 100, 50 μ g/mL) diluted in pH 7.6 TBS (50 mM Tris-Cl pH 7.5, 150 mM sodium chloride) and 0.05% Tween 20 (TBS-Tween). After the antigen was blotted, the membrane was allowed to dry before adding Blocking Buffer supplemented with 0.05% Tween 20. The membrane was allowed to block for one hour on a rocking platform at room temperature. After blocking, the membrane was washed 3 times with TBS-Tween for 5 minutes per wash. The membrane was then incubated for 1 hour at room temperature on a rocking platform in a 10% solution of SBI, diluted 1:100 in TBS-Tween, after which it was washed 5 times with TBS-Tween for 5 minutes per wash. The membrane was then incubated with 25 mL of 1:25,000 sheep anti-bovine IgG-HRP detection antibody in TBS-Tween for 1 hour at room temperature on the rocking platform and then washed 5 times for 5 minutes per wash. SuperSignal Pico Chemiluminescent substrate was added (4 mL of each kit reagent), and the membrane was scanned on the LICOR using the High Resolution Setting. The resulting chemiluminescent image was analyzed using ImageStudio.

SBI–Antigen Binding: ELISA

96-well medium binding plates were coated overnight at 4°C with 100 μ L of 5 μ g/mL or 10 μ g/mL antigen in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Each well was washed 5 times with TBS-Tween. Plates were then blocked for 1 hour at room temperature using 300 μ L Blocking Buffer and 0.05% Tween. Wells were washed again with TBS-Tween and then incubated with 100 μ L SBI or denatured SBI in TBS-Tween for 1 hour at room temperature. Afterward, the wells were emptied and washed 5 times with TBS-Tween. Each well received 100 μ L of sheep anti-bovine IgG-HRP added at 1:125,000 or 1:150,000 dilution in TBS-Tween and incubated for 1 hour at room temperature. Plates were again washed 5 times with TBS-Tween. The reaction was visualized by the addition of 100 μ L of TMB substrate and stopped with the addition of 100 μ L 0.2 M sulfuric acid. Absorbance was read at 450 nm using a microplate reader (Molecular Devices, Sunnyvale, CA).

Control experiments were performed for each antigen specific ELISA to determine the non-specific binding of the detection antibodies and the specific recognition of the antigens by IgG contained in SBI. Non-specific binding of the detection antibodies was tested by running the ELISA without the SBI addition step. Blocking effectiveness was tested to demonstrate specificity of SBI IgGs for the test antigens by comparing the absorbance in unblocked wells to wells not coated with antigen. Functional binding of IgG for antigens was determined by comparing SBI treated wells to wells treated with a denatured SBI control. Overall binding specificity of SBI IgGs for the antigen was determined by a comparison of the complete ELISA with SBI to the

denatured SBI control and the ELISA run without antigen but with blocking of the wells. The full list of controls is shown below.

- (1) Binding control: antigen, **no block**, SBI, detection antibody, substrate
- (2) Blocking control: **no antigen**, block, SBI, detection antibody, substrate
- (3) Non-specific surface binding control: **no antigen, no block**, SBI, detection antibody, substrate
- (4) Detection antibody background control: antigen, block, **no SBI**, detection antibody, substrate
- (5) SBI background control: antigen, block, SBI, **no detection antibody**, substrate
- (6) Functional binding control: antigen, block, **denatured SBI**, detection antibody, substrate

RESULTS

Dot Blot

The binding of *C. albicans* lysate, Als3, *H. pylori* lysate, CagA, Shiga-like toxin 1, aflatoxin M1, and CDT antigens by IgG in SBI were initially screened by a dot blot method. All antigens except the *H. pylori* lysate and aflatoxin M1 exhibited binding by dot blot. No additional testing was performed on the *H. pylori* lysate, but aflatoxin M1 antigen-IgG binding tested negative by ELISA (Appendix).

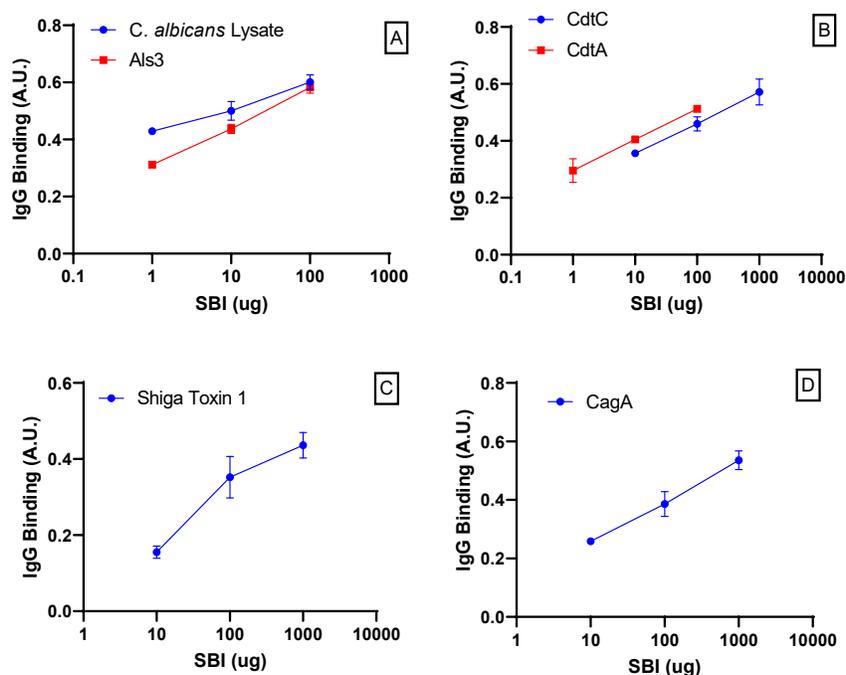


Figure 1. SBI binding affinity to various antigens A) SBI-IgG binds to *C. albicans* antigens, B) SBI-IgG binds to CDT antigens, C) SBI- IgG binds to Shiga-like toxin 1, D) SBI-IgG binds to a *H. pylori* CagA antigen. Error bars represent \pm one standard deviation from triplicate data. Absorbance units (A.U.) measured at 450 nm.

ELISA

To demonstrate specificity of IgG binding, antigens were tested using a modified ELISA demonstrated in (Fig. 1, A-D). Absorbance of IgG bound to immobilized antigens increased proportionally to SBI concentration. SBI IgG specifically binds *C. albicans* lysate, *C. albicans* Als3 protein, CDT subunit A, CDT subunit B, Shiga-like toxin 1, and *H. pylori* CagA protein.

Specificity of immunoglobulin binding to each antigen was assessed by comparing the “Complete ELISA” curves shown in (Fig. 2, A-F) to control curves which excluded steps throughout the modified ELISA. SBI IgG binds specifically to antigens (Complete ELISA) when compared to the binding controls (Fig. 2, A-F). The complete ELISA displayed a higher absorbance compared to the blocking control (No antigen, Block), demonstrating IgG specificity for the antigen. Effective blocking is demonstrated by comparing wells treated with blocking buffer (No antigen, Block) to wells without blocking buffer (No antigen, No block). In summary, results from the specificity and binding controls indicate IgG binds specifically to each antigen in Table 1.

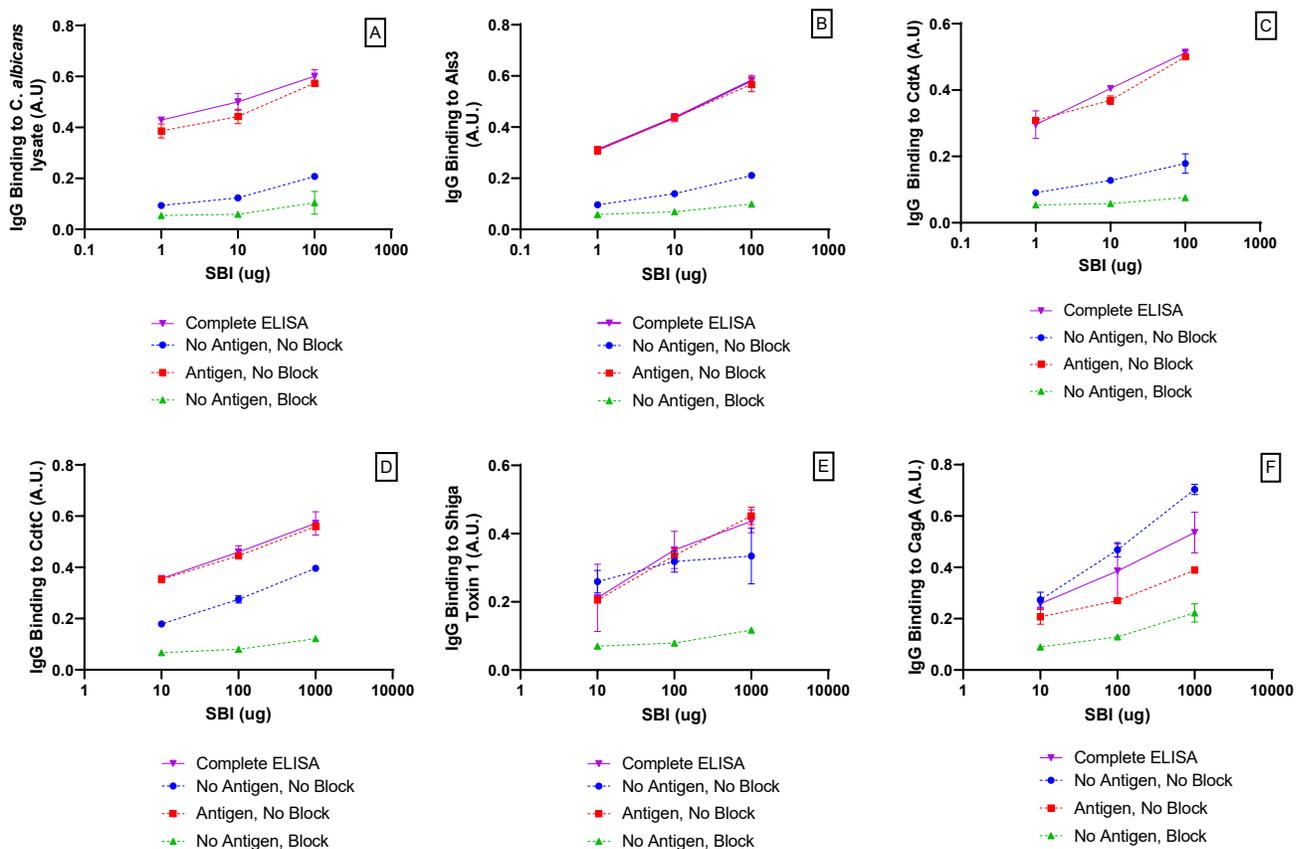


Figure 2. SBI IgG had a higher affinity for antigen protein (Complete ELISA) when compared to the blocking protein (- antigen, +block). Effective blocking is demonstrated by comparing wells treated with blocking buffer (-antigen, +block) to wells not treated with blocking buffer (-antigen, -block) A) SBI IgG binding to *C. Albicans*, B) SBI IgG binding to Als3, C) SBI IgG binding to CdtA, D) SBI IgG binding to CdtC, E) SBI IgG binding to Shiga Toxin 1, F) SBI IgG Binding to CagA. Error bars represent \pm one standard deviation from triplicate data. Absorbance units (A.U.) measure at 450 nm.

Functional binding of SBI was assessed by comparing the complete ELISA curves shown in (Fig. 3, A-F) to the denatured SBI control, which displayed no binding activity for each ELISA. The background controls, no addition of the detection antibody or SBI, showed no significant absorbance compared to the Complete ELISA curve.

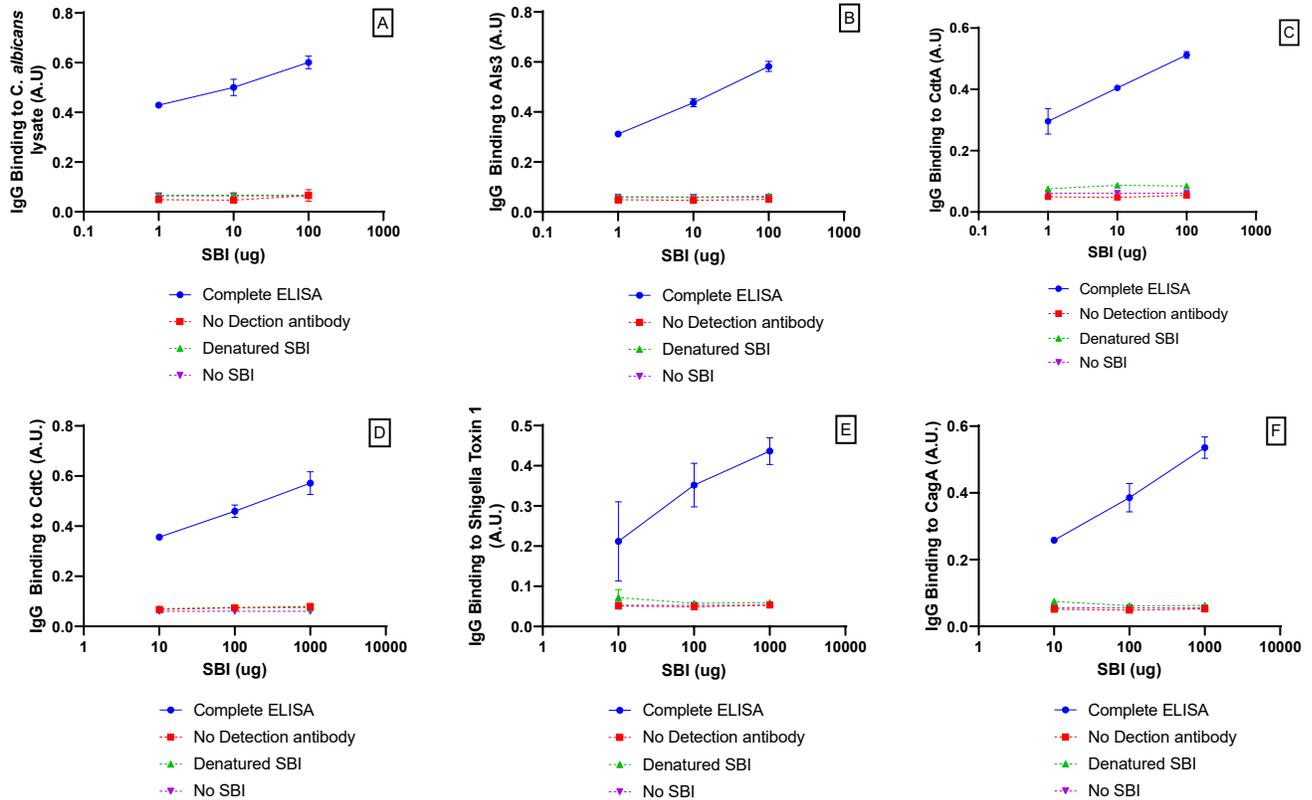


Figure 3. Specificity control experiments IgG binding to A) *C. albicans* lysate, B) *C. albicans* Als3 antigen, C) CdtA, D) CdtC, E) Shiga-like toxin 1, and F) *H. pylori* CagA antigen. Error bars represent ± one standard deviation from triplicate data. Absorbance units (A.U.) measured at 450 nm. Absorbance units (A.U.) measured at 450 nm

DISCUSSION

The IgGs contained in SBI specifically bind *C. albicans* lysate and Als3 protein, CDT subunits A and C, Shiga-like toxin 1 subunit B, and *H. pylori* CagA protein as determined by ELISA. While the dot blots for *H. pylori* lysate and aflatoxin M1 showed no binding that does not prove a lack of SBI binding to those antigens. The lack of binding to *H. pylori* lysate conflicts with the work by Horgan *et al* have previously demonstrate binding of Immunolin IgGs to *H. pylori* lysate¹¹. The discrepancy in the two sets of data is likely due to strain differences between the *H. pylori* lysates. With the strain in this study likely having less CagA expression or recovery as binding of IgG to the purified CagA antigen was demonstrated by ELISA. The case of the aflatoxin dot blot results presents a different challenge. Aflatoxins are small molecules which are present problems for a traditional sandwich ELISA format due to presentation issues to the IgG molecules resulting from steric hinderances. To overcome this issue, a conjugate of BSA and aflatoxin-M1 was bound to the PVDF membrane

and used for an ELISA (data not shown) which showed no SBI IgG binding. It is still likely that SBI can bind some mycotoxins as BSA has been shown to bind to alternariol mycotoxin and BSA is present in SBI¹².

Conclusion

The immunoglobulin content of SBI has previously been shown to bind to a variety of microbial antigens (e.g., LPS, flagellin, peptidoglycan, etc) associated with gastrointestinal disorders. To continue to understand the broad impact of SBI, new relevant antigens of interest were chosen to test for IgG binding. The results presented here suggest SBI can bind to antigenic components from negative-gram bacteria (*C. albicans*, *H. pylori*, *S. dysenteriae*, and *E. coli*) commonly associated with GI inflammation and disease.

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APPENDIX

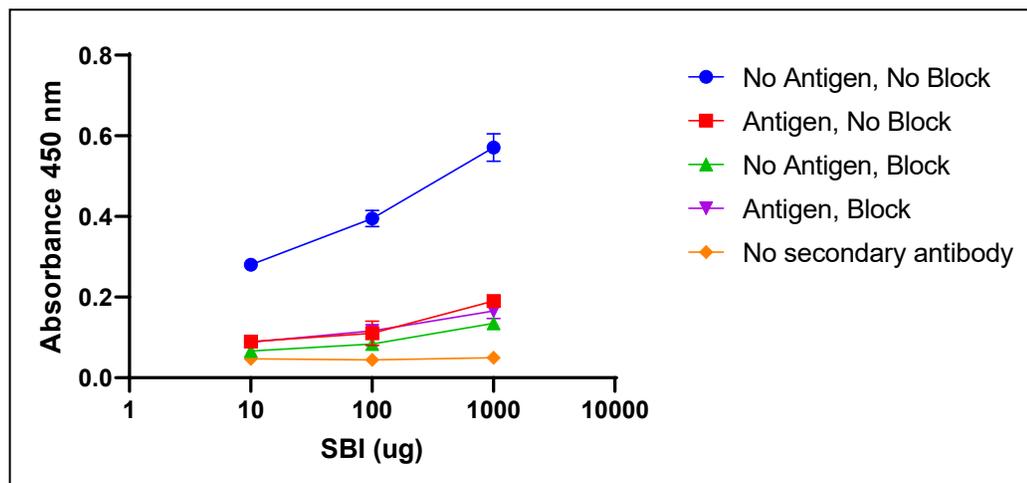


Figure 4. SBI complete ELISA (Antigen, Block) showed no significant absorbance above the No Antigen, Block control indicating no IgG binding against the Aflatoxin M1 antigen.