

Group No.	Antigen Received	Number of Mice	Antigen Delivery Method
Group 1	Chitosan-coated IT protein	7 mice	Oral administration
Group 2	Chitosan-coated CIT protein	7 mice	Oral administration
Group 3	Chitosan-coated CtxB protein	5 mice	Oral administration
Group 4	Empty chitosan	5 mice	Oral administration
Group 5	Food and water only	5 mice	No intervention
Group 6	IT protein + Freund's adjuvant	5 mice	Subcutaneous injection
Group 7	Freund's adjuvant	5 mice	Subcutaneous injection

Table S1. Experimental design and grouping of mice used for immunization studies. Animals received oral administration of chitosan-encapsulated recombinant antigens (IT, CIT, or CtxB), empty chitosan nanoparticles, or subcutaneous immunization with IT protein plus Freund's adjuvant. Untreated and adjuvant control groups were included

Tissue	Group	Histopathological findings	Final score	Significance vs control
Cecum	CIT	Mild or no epithelial injury and inflammatory infiltration	0.5 ± 0.3	$P = 0.0237$
	IT+Freund	Moderate inflammatory infiltration and crypt irregularity	1.5 ± 0.4	ns
	IT	Mild to moderate epithelial and crypt alterations	1.0 ± 0.3	ns
	Control	Severe epithelial disruption, inflammatory infiltration, and crypt damage	2.5 ± 0.5	—
Colon	CIT	Preserved epithelial continuity with minimal inflammation	0.3 ± 0.2	$P = 0.0215$
	IT+Freund	Mild to moderate inflammatory infiltration and crypt irregularity	1.4 ± 0.4	ns
	IT	Mild epithelial and crypt alterations	1.0 ± 0.3	ns
	Control	Severe epithelial disruption and inflammatory infiltration	2.6 ± 0.5	—
Kidney	CIT	Normal renal histology without detectable injury	0.0 ± 0.0	$P = 0.0190$
	IT+Freund	Mild tubular and interstitial alterations	1.0 ± 0.3	ns
	IT	Mild renal structural alterations	0.8 ± 0.2	ns
	Control	Tubular degeneration and marked interstitial inflammation	2.0 ± 0.4	—

Table S2. Histopathological scoring of cecum, colon, and kidney tissues in different experimental groups following *EHEC* challenge. Scoring scale: 0 = no lesion, 1 = mild, 2 = moderate, 3 = severe tissue damage. Statistical comparisons were performed using the Kruskal–Wallis test followed by Dunn's multiple-comparisons test; only the CIT group showed a statistically significant difference from the control group across all three tissues ($P < 0.05$). ns = not significant; — = reference group

Group	Replicate 1 (cm)	Replicate 2 (cm)	Replicate 3 (cm)	Mean ± SD	Significance vs Control
CIT	11.2	11.0	10.9	11.0 ± 0.2	***
IT	9.8	9.5	9.5	9.6 ± 0.2	*
IT+Freund	10.5	10.0	10.3	10.3 ± 0.3	**
Control	8.5	8.6	8.2	8.4 ± 0.2	—
CtxB	8.4	8.2	8.8	8.5 ± 0.3	ns
Freund	8.4	8.8	8.0	8.4 ± 0.4	ns
PBS+Chitosan	8.2	8.5	7.9	8.2 ± 0.3	ns

Table S3. Colon length measurements (cm) across all experimental groups following *EHEC* challenge. Statistical comparisons were performed using one-way ANOVA followed by Dunnett's T3 multiple-comparisons test. Asterisks indicate significance versus the control group (*P < 0.05, **P < 0.01, ***P < 0.001); ns = not significant; — = reference group.

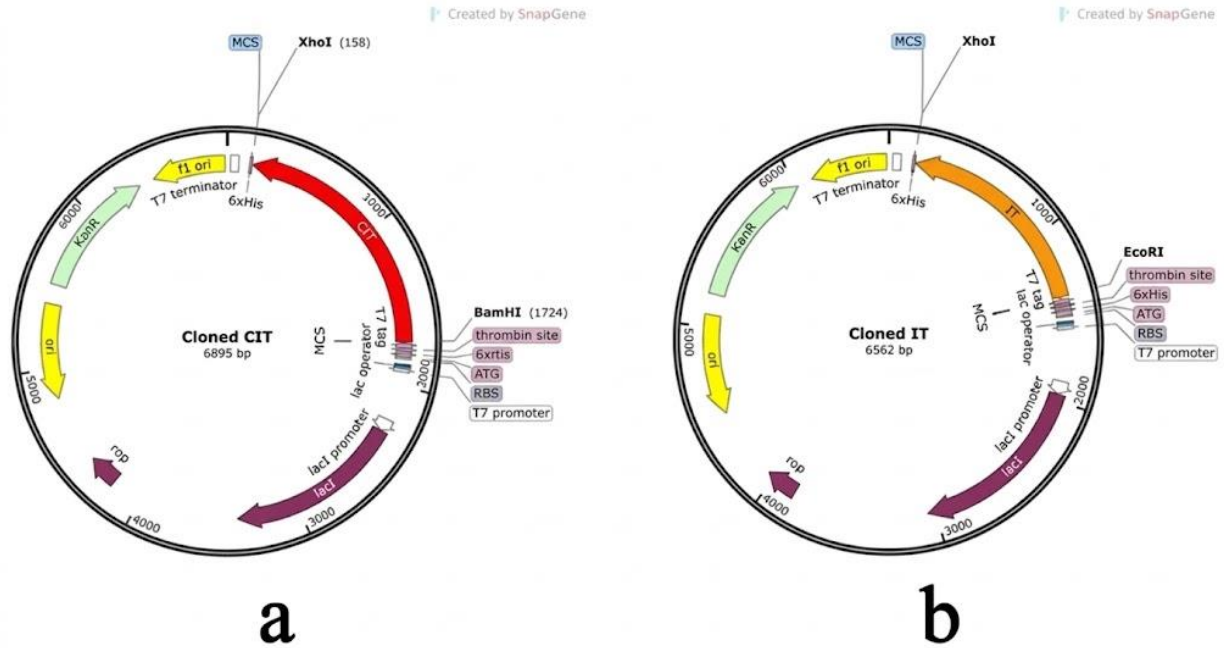


Fig. S1. Schematic map of the recombinant expression vectors. (a) Plasmid map of pET-28a(+) carrying the CIT gene insert (6895 bp), inserted between the *Bam*HI and *Xho*I restriction sites. (b) Plasmid map of pET-28a(+) carrying the IT gene insert (6562 bp), inserted downstream of the T7 promoter near the *Xho*I restriction site. Both recombinant constructs incorporate N-terminal and C-terminal 6×His tags, a kanamycin resistance gene (*Kan*^R), and a T7 promoter system for expression. Plasmid maps were generated using SnapGene software.

Intimin-tir:

```
tttgatcagacaaagcgagcattaccgaaataaagcggataaaaccaccgcgggtgcgaacggcaaaagatgcgattaaatataccgtgaaagtgatgaaaaacggccagccgggtgaac
aaccagagcgtgaccttttagcaccaactttggcatgtttaacggcaaaagccagaccagcgaccaccggcaacgatggcggcgaccattacctgaccagcagcagcgccggttaa
agcaaccgtgagcgcaaccgtgagcgatgggtgcagaagtgaagcgaccgaagtgaccttttcgatgaactgaaattgatacaaaagtgatattatggcaacaacgtgcgtggcgaa
ctgccgaacatttggctgcagtatggccagtttaactgaaagcgagcggcgcgatggcacctatagctgtatagcgaaaacaccagcattgcgaccgtggatgcgagcggcaaaagt
acctgaacggcaaaagcgagcgtggtgattaaagcgaccagcgcgataaacagaccgtgagctataccattaaagcggcgagctatatgattaaagtggataaacaggcgctattatcgcg
atgcgatgagcatttgcataaacctgctgcgagcaccagaccgtgctgagcgatattatgatagctggggcgcgcggaacaaatagccattatagcagcatgaacagcattaccgcg
tgattaaacagaccagcaggaacagcgtagcggcgtagcagcacctataacctgattaccagaaccgcgtccggggcggtgaacgtgaacacccgaacgtgtatgcgggtgctgct
ggaagaagccgcagcgaaagaagccgcgccaaagaagcggccgaagaagcagcagcaaaaccggaaccggatagcccgaccaccaccgatccggatgcagcagcaagcg
caaccgaaaccgcaaccgtgatcagctgaccaagaagcgtttcagaacccggataaccgaaagtgaacattgatgaactgggcaacgcgattccgagcggcgctgctgaaagatgat
gtggtggcgaacattgaagaacaggcgaaagcggcgggcggaagaagcgaacagcagcgattgaaacaacgcgcagcgcgagaaaaatgatgaacagcagcgcaaacgtc
aggaagaactgaaagtgcagcggcgcggtgctaa
```

CtxB-Intimin-Tir (CIT):

```
GGATCCATGACCCCGCAAAACATTACTGACCTGTGCGCTGAATATCATAACACCCAAATTCACACCCTGAACGATAA
AATCTTCTCTTACACCGAATCCCTGGCGGGTAAGCGTGAAATGGCTATCATCACCTTCAAAAACGGCGCGACTTTTC
AGGTGGAAGTTCCGGGTAGCCAGCACATCGACTCCCAGAAGAAAGCAATTGAACGTATGAAAGATACCTGCGCAT
CGCATACCTGACCGAAGCGAAAAGTTGAAAACTGTGCGTTTGAACAACAAGACCCCGCATGCCATCGTGCTATT
TCTATGGCTGCAGAAGCTGCCGCAAAAGCTGAATTCTTTGATCAGACCAAAGCGAGCATTACCGAAATTAAAGCGG
```

ATAAAACCAACCGCGGTGGCGAACGGCAAAGATGCGATTAAATATACCGTGAAAGTGATGAAAAACGGCCAGCCGGT
GAACAACCAGAGCGTGACCTTTAGCACCAACTTTGGCATGTTTAACGGCAAAAGCCAGACCCAGGCGACCACCGG
CAACGATGGCCGTGCGACCATTAACCTGACCAGCAGCAGCGCCGGTAAAGCAACCGTGAGCGCAACCGTGAGCGA
TGGTGCAGAAGTGAAAGCGACCGAAGTGACCTTTTTTCGATGAACTGAAAATTGATAACAAAAGTGATATTATTGGCA
ACAACGTGCGTGGCGAACTGCCGAACATTTGGCTGCAGTATGGCCAGTTTAAACTGAAAGCGAGCGGCGGCGATGG
CACCTATAGCTGGTATAGCGAAAACACCAGCATTGCGACCGTGGATGCGAGCGGCAAAGTGACCCTGAACGGCAAA
GGCAGCGTGGTGATTAAAGCGACCAGCGGCGATAAACAGACCGTGAGCTATACCATTAAAGCGCCGAGCTATATGAT
TAAAGTGGATAAACAGGCGTATTATGCGGATGCGATGAGCATTTGCAAAAACCTGCTGCCGAGCACCCAGACCGTGC
TGAGCGATATTTATGATAGCTGGGGCGCGGCGAACAATATAGCCATTATAGCAGCATGAACAGCATTACCGCGTGGA
TTAAACAGACCAGCAGCGAACAGCGTAGCGGCGTGAGCAGCACCTATAACCTGATTACCCAGAACCCGCTGCCGGG
CGTGAACGTGAACACCCCGAACGTGTATGCGGTGTGCGTGGAAGAAGCCGCAGCGAAAGAAGCCGCGGCCAAAG
AAGCCGCCGCCAAAGAAGCAGCAGCAAAAACCGGAACCGGATAGCCCGACCACCACCGATCCGGATGCAGCAGCAA
GCGCAACCGAAACCGCAACCCGTGATCAGCTGACCAAAGAAGCGTTTCAGAACCCGGATAACCAGAAAAGTGAACA
TTGATGAACTGGGCAACGCGATTCCGAGCGGCGTGCTGAAAGATGATGTGGTGGCGAACATTGAAGAACAGGCGA
AAGCGGCGGGCGAAGAAGCGAAACAGCAGGCGATTGAAAACAACGCGCAGGCGCAGAAAAAATATGATGAACAG
CAGGCGAAACGTCAGGAAGAACTGAAAGTGAGCAGCGGCGCGGGCTAACTCGAG

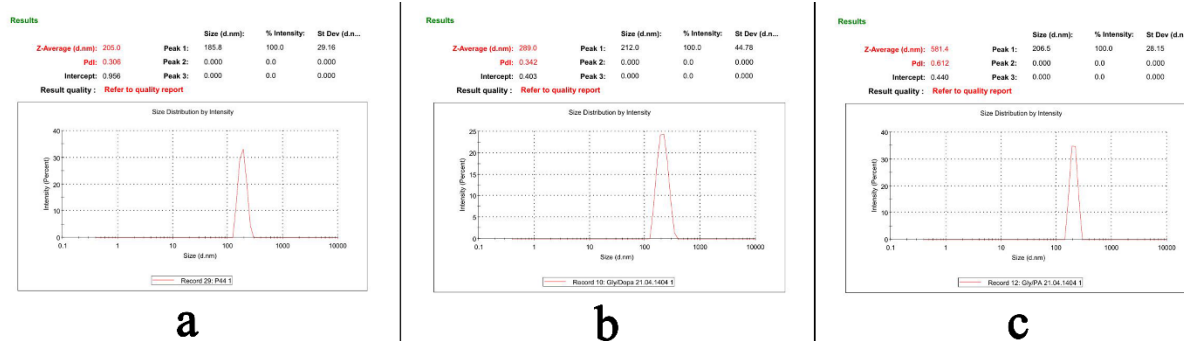
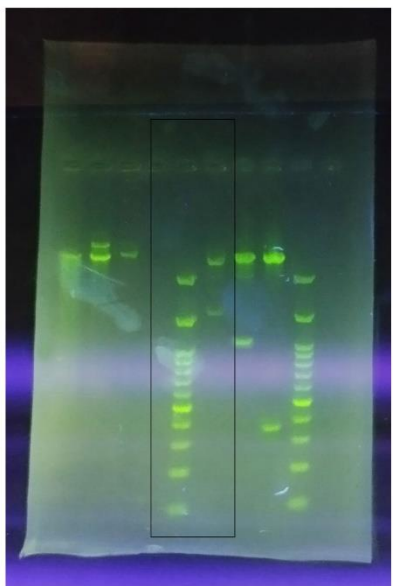
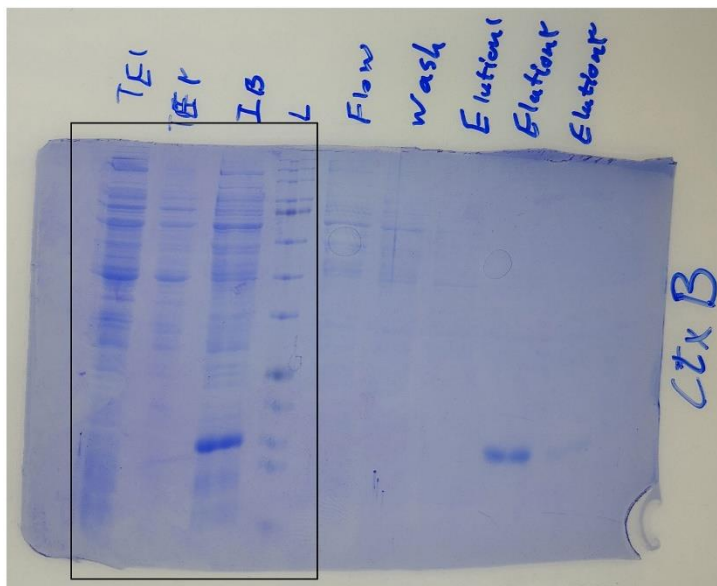


Fig. S2. Dynamic light scattering (DLS) analysis of chitosan nanoparticle formulations encapsulating recombinant antigens.

Representative DLS intensity distribution profiles and physicochemical parameters of chitosan nanoparticles loaded with recombinant antigens. (a) CIT-loaded nanoparticles, (b) IT-loaded nanoparticles, and (c) CtxB-loaded nanoparticles. Mean particle size, polydispersity index (PDI), and Z-average values are summarized for each formulation, indicating differences in particle size distribution and colloidal homogeneity among the three systems.



a



b

Fig. S3. Full-length gel images corresponding to Fig. 1. Full-length gel images corresponding to the cropped regions presented in Fig. 1a and Fig. 1b are shown. The regions outlined by the black rectangles indicate the exact portions of the full-length gel/blot images that were cropped and presented in the corresponding panels of Fig. 1.

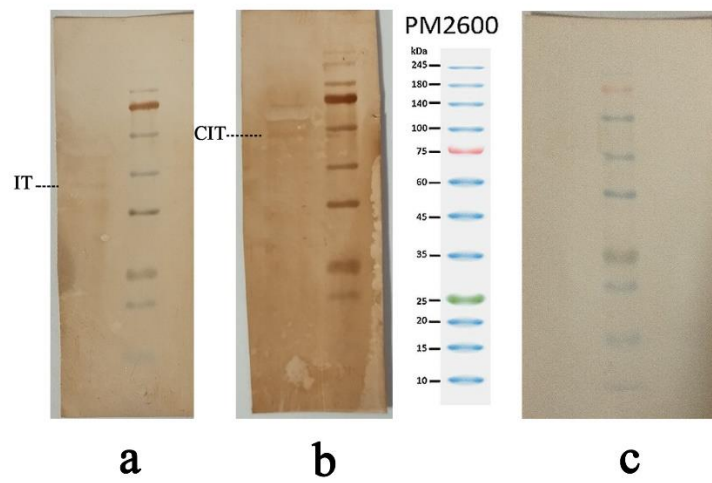


Fig. S4. Western blot analysis demonstrating seroconversion in immunized mice. Recombinant proteins were probed with pooled sera collected from vaccinated or control animals. (a) Recognition of recombinant IT protein by sera from IT-immunized mice. (b) Recognition of recombinant CIT protein by sera from CIT-immunized mice. (c) Lack of detectable reactivity in sera collected from control mice. The presence of specific immunoreactive bands in vaccinated groups and their absence in control sera confirms seroconversion and antigen-specific antibody recognition following immunization.

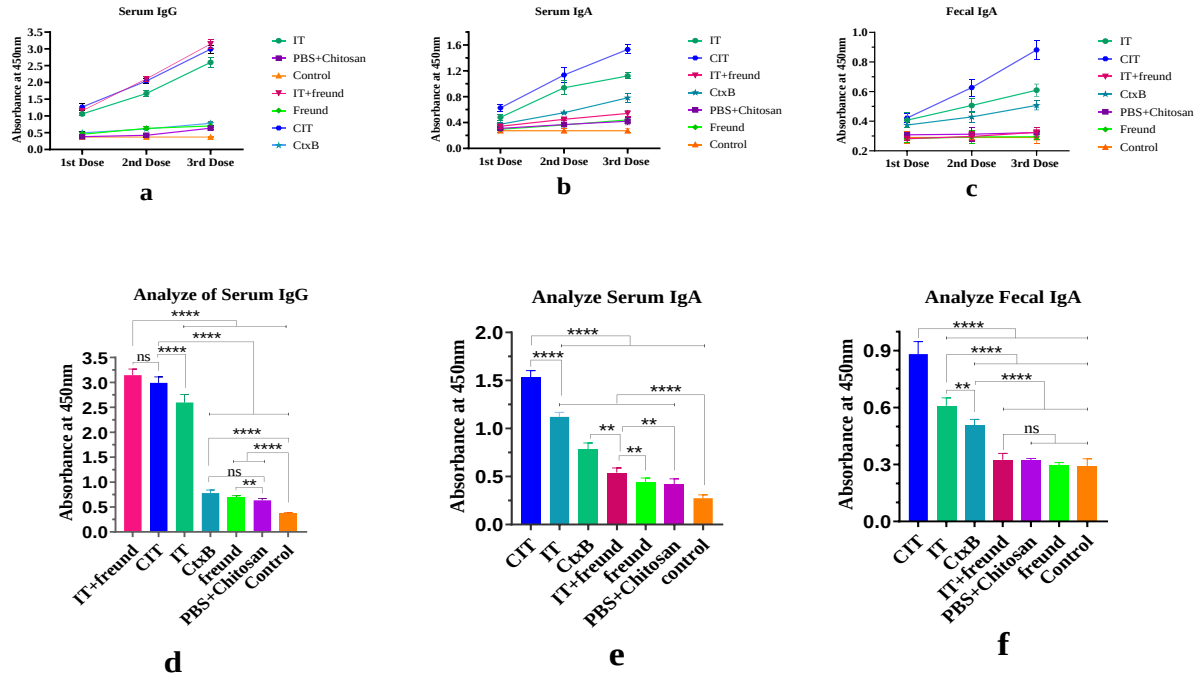


Fig. S5. Humoral immune responses induced by different vaccine formulations. (a–c) Kinetics of antigen-specific antibody responses following three immunization doses, showing serum IgG (a), serum IgA (b), and fecal secretory IgA (c) levels measured at different time points. (d–f) Comparison of antibody responses after the third immunization at a dilution of 1:250 for serum IgG (d), and 1:20 for serum IgA (e), and fecal secretory IgA (f) levels across all experimental and control groups. Data are presented as mean $\pm$ SD. Statistical significance is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

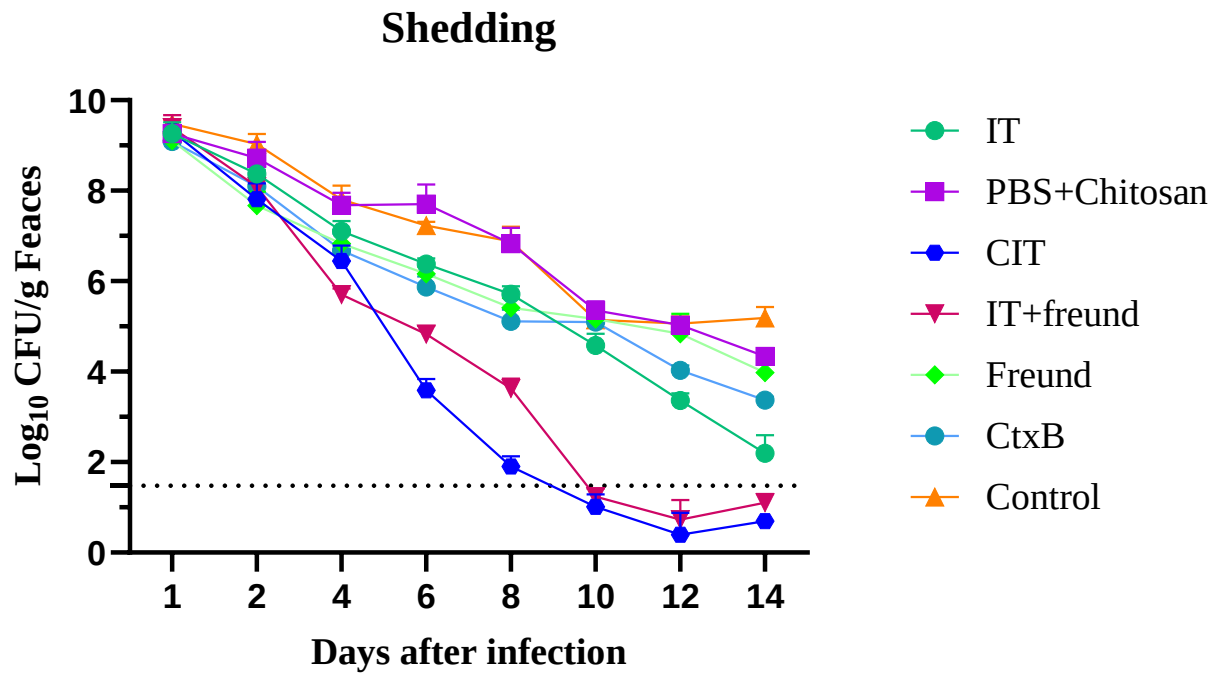


Fig. S6. Comprehensive analysis of fecal shedding following oral challenge with *Escherichia coli* O157:H7. Fecal samples were collected at the indicated time points every other day over 14 days post-challenge and cultured on CT-SMAC agar following enrichment. Results are presented as mean $\pm$ SEM and expressed as log₁₀ CFU/g feces. The horizontal dashed line indicates the detection limit of the assay (30 CFU/g). The figure includes all vaccine, adjuvant-only, nanoparticle-only, and untreated control groups.

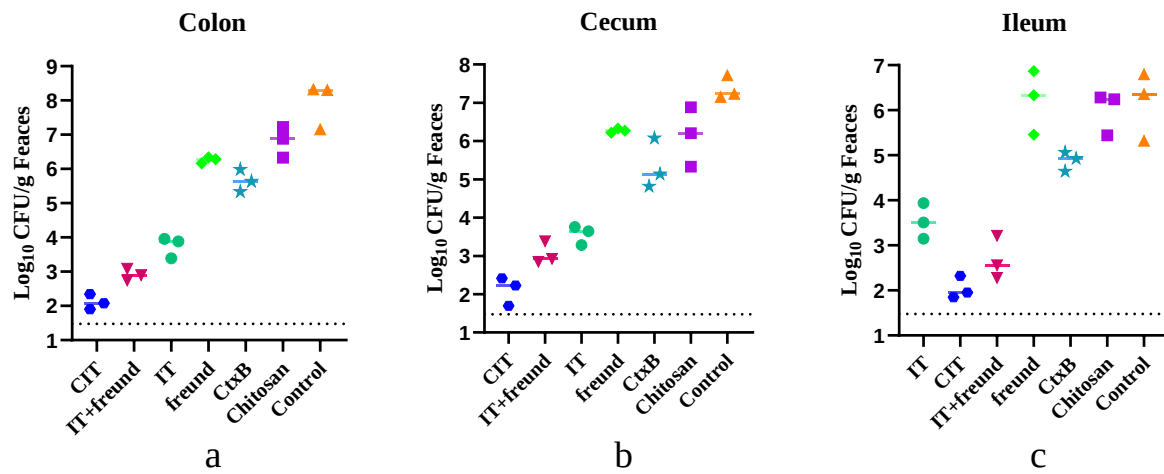


Fig. S7. Comprehensive analysis of organ bacterial colonization following *EHEC* O157:H7 challenge. Bacterial loads in the (a) colon, (b) cecum, and (c) small intestine were determined by CFU enumeration on CT-SMAC agfollowing tissue homogenization and serial dilution. Results are presented as mean $\pm$ SEM and expressed as log₁₀ CFU/g tissue. The figure includes all experimental and control groups. Bacterial counts in the spleen and liver remained below the assay detection limit in all experimental groups, while only limited bacterial recovery was detected in kidneys from control animals.

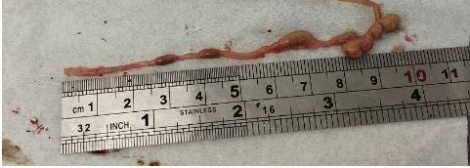
Groups	Colon length
IT	
CIT	
IT+ Freund	
CtxB	
Freund	
Control	
Chitosan+PBS	

Fig. S8. Evaluation of mouse colon length across experimental groups. A representative photographic comparison of freshly isolated mouse colons from the seven study groups. A standard metric ruler is placed alongside the samples for scale to assess colon length shortening as an indicator of disease severity. In comparison to the healthy control group, experimental groups subjected to disease induction show varying degrees of significant colon shortening. The average measurements and statistical analysis for all animals are presented in the main text.