Table S1. Oligonucleotides used for cloning toxin domains of *C. difficile* VPI10463

Toxin and fragment	Primer	Sequence[b]	Amino acids[a]
Toxin A fragment 1	TxA-frag1-Fw	agccatgggtttaatatctaaagaagagttaataaaactcgcatatagc	1 - 669
	TxA-frag1-Rv	ccggatccacttaatctagcaaattcgcttgtgttgaattcatctttacc	
Toxin A Fragment 2	TxA-frag2-Fw	agccatgggcgtaacctttattggacatggtaaagatgaattcaacacaagcgaatttgctag	649 - 1265
	TxA-frag2-Rv	ccggatccgtaaaatttacctgggtataaatctcttattgaatcaagtaatc	
Toxin A fragment 3	TxA-frag3-Fw	agccatgggcgaaaatgacggaactagattacttgattcaataagagatttatacccagg	1245 - 1848
	TxA-frag3-Rv	ccggatccaaattctataggatcaaaatagaataatgaattatttatattgattaatcc	
Toxin A fragment 4	TxA-frag4-Fw	agccatgggcttagttaaaggattaatcaatataaataattcattattctattttgatcc	1828 - 2710
	TxA-frag4-Rv	ccggatccgccatatatcccaggggcttttactccatcaacacc	
Toxin B fragment 1	TxB-frag1-Fw	agccatgggcttagttaatagaaaacagttagaaaaaatggcaaatgtaag	1 - 602
	TxB-frag1-Rv	ggctcgagcttacctaggcggttcatctctatgtctgtataggatccaggagtctttgcaaataagttacatgctgcttc	
Toxin B fragment 2	TxB-frag2-Fw	agccatgggccagttacaaggagataaaattagttatgaagcagcatg	582 -1194
	TxB-frag2-Rv	ccggatcctgttattgatggtgctgaaaagaagtgatctatatcatcag	
Toxin B fragment 3	TxB-frag3-Fw	agccatgggcggttcaggtcatactgtaactgatgatatagatcacttc	1174 - 1786
	TxB-frag3-Rv	ccggatccatcactaaagttaaaagatagcttatttgccaaagtcttatc	
Toxin B fragment 4	TxB-frag4-Fw	agccatgggcttcgttaatgtttttaaagataagactttggcaaataagc	1766 - 2366
	TxB-frag4-Rv	ccggatccttcactaatcactaattgagctgtatcaggatcaaaataatac	

[a] Corresponding amino acids sequence of toxin cloned on fragment. All toxin fragments are cloned to give a 20 amino acid overlap between adjacent toxin domains.

[b] Underlined sequence indicates the restriction sites introduced for cloning.

Table S2. Strains and plasmids used in study

Strain or plasmid	Relevant properties	Reference or source
Plasmids		
pKA432	pET28a with *C. difficile* toxin A catalytic domain (fragment 1)	This work
pKA433	pET28a with *C. difficile* toxin A translocation domain (fragment 2)	This work
pKA434	pET28a with *C. difficile* toxin A translocation domain (fragment 3)	This work
pKA435	pET28a with *C. difficile* toxin A receptor binding domain (fragment 4)	This work
pKA436	pET28a with *C. difficile* toxin B catalytic domain (fragment 1)	This work
pKA437	pET28a with *C. difficile* toxin B translocation domain (fragment 2)	This work
pKA438	pET28a with *C. difficile* toxin B translocation domain (fragment 3)	This work
pKA439	pET28a with *C. difficile* toxin B receptor binding domain (fragment 4)	This work
pAF100	Expression plasmid for secreted expression in lactobacilli	(31)
pAF900	Expression plasmid for cell wall anchored expression in lactobacilli	(31)
pKA498	pAF100 with VHH-B2 inserted for secreted expression	This work
pKA452	pAF100 with VHH-E2 inserted for secreted expression	This work
pKA453	pAF100 with VHH-G3 inserted for secreted expression	This work
pKA500	pAF100 with VHH-D8 inserted for secreted expression	This work
pKA456	pAF100 with VHH-G1 inserted for cell wall anchored expression	This work
pKA458	pAF100 with VHH-D2 inserted for cell wall anchored expression	This work
pKA461	pAF100 with VHH-B5 inserted for cell wall anchored expression	This work
pKA463	pAF100 with VHH-G9 inserted for cell wall anchored expression	This work
pKA457	pAF100 with VHH-B2 inserted for cell wall anchored expression	This work
pKA459	pAF100 with VHH-E2 inserted for cell wall anchored expression	This work
pKA460	pAF100 with VHH-G3 inserted for cell wall anchored expression	This work
pKA462	pAF100 with VHH-D8 inserted for cell wall anchored expression	This work
Strains		
KKA370	*E. coli* BL21 with plasmid pKA432	This work
KKA371	*E. coli* BL21 with plasmid pKA433	This work
KKA372	*E. coli* BL21 with plasmid pKA433	This work
KKA373	*E. coli* BL21 with plasmid pKA434	This work
KKA374	*E. coli* BL21 with plasmid pKA435	This work

KKA375	*E. coli* BL21 with plasmid pKA436	This work
KKA376	*E. coli* BL21 with plasmid pKA437	This work
KKA377	*E. coli* BL21 with plasmid pKA438	This work
L. paracasei BL23	Previously considered a plasmid-free *L. casei* 393 strain	(23)
KKA101	*L. paracasei* BL23 harbouring empty expression plasmid	(33)
KKA382	*L. paracasei* BL23 with plasmid pKA453, secreted VHH-G3	This work
KKA440	*L. paracasei* BL23 with plasmid pKA452, secreted VHH-E2	This work
KKA441	*L. paracasei* BL23 with plasmid pKA500, secreted VHH-D8	This work
KKA442	*L. paracasei* BL23 with plasmid pKA498, secreted VHH-B2	This work
KKA412	*L. paracasei* BL23 with plasmid pKA457, cell wall anchored VHH-G1	This work
KKA414	*L. paracasei* BL23 with plasmid pKA457, cell wall anchored VHH-D2	This work
KKA417	*L. paracasei* BL23 with plasmid pKA457, cell wall anchored VHH-B5	This work
KKA419	*L. paracasei* BL23 with plasmid pKA457, cell wall anchored VHH-G9	This work
KKA413	*L. paracasei* BL23 with plasmid pKA457, cell wall anchored VHH-B2	This work
KKA415	*L. paracasei* BL23 with plasmid pKA459, cell wall anchored VHH-E2	This work
KKA416	*L. paracasei* BL23 with plasmid pKA460, cell wall anchored VHH-B3	This work
KKA418	*L. paracasei* BL23 with plasmid pKA462, cell wall anchored VHH-D8	This work
C. difficile 630 (tcdA-,tcdB+)	Toxin A negative and toxin B positive strain of *C. difficile* 630Δerm	(8)
E. coli DH5α		
E. coli BL21-CodonPlus(DE3)-RIPL		

Table S3. Progression of infection in hamsters

Groups	Hamster no	1. Day	2. Day	3. Day	4. Day	5. Day
Spores only	58-1	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	58-2	GDH negative - -	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death
	58-3	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	58-4	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	58-5	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	58-6	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
$5x10^9$ CFU KKA413 and $5x10^9$ CFU KKA416	54-1	GDH negative - -	GDH negative - -	GDH positive Toxin negative Wet tail	GDH positive Toxin negative Wet tail	N.D Toxin negative Wet tail
	54-2	GDH negative - -	GDH positive N.D -	GDH positive Toxin negative Wet tail	GDH positive Toxin negative Wet tail	N.D Toxin negative Wet tail
	54-3	GDH negative - -	GDH negative - -	GDH positive Toxin negative Wet tail	GDH positive Toxin positive Wet tail	Death
	54-4	GDH negative - -	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death
	54-5	GDH negative - -	GDH negative - -	GDH negative - -	GDH positive Toxin negative Wet tail	GDH positive Toxin positive Wet tail
	54-6	GDH negative - -	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death
$1x10^{10}$ CFU KKA101	53-1	GDH negative - -	GDH negative - -	GDH negative - -	GDH positive Toxin positive - Death -	
	53-2	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	53-3	GDH negative - -	GDH negative - -	GDH positive Toxin negative Wet tail	GDH positive Toxin positive Wet tail	Death
	53-4	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	53-5	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	
	53-6	GDH negative - -	GDH negative - -	GDH positive Toxin positive Wet tail	Death	

Markers for the progression of infection for hamsters receiving: *C. difficile* spores only; *C. difficile* spores and engineered *L. paracasei* BL23 expressing cell wall anchored anti-toxin B neutralizing VHH fragments B2 and G3 (strain KKA413 and KKA416); or *C. difficile* spores and non-expressing *L. paracasei* BL23 (strain KKA101). Glutamate Dehydrogenase (GDH) was measured by ELISA in fecal droppings as a marker for spore germination and presence of vegative *C. difficile*. Presence of toxins in the feces were detected by an ELISA for toxin B as marker of onset of virulence. Episodes of diarrhea were observed through the presence of wet tail. (-) No sample taken as feces were still GDH negative. (ND) measurement not done.

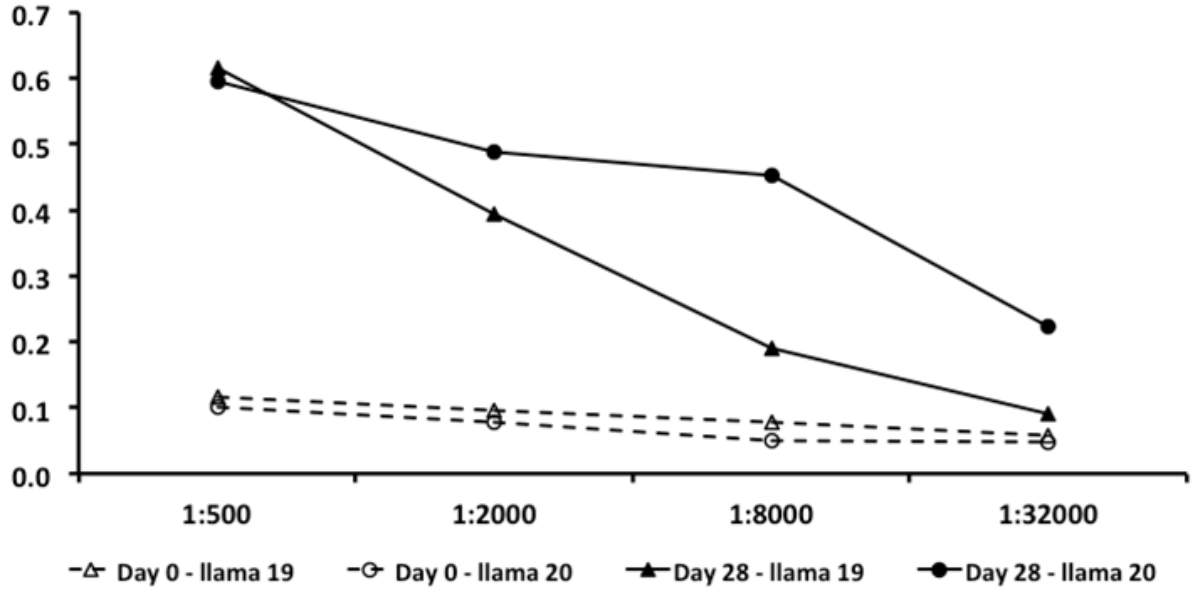

Figure S1. Analysis of serum antibody responses in llamas immunized with *C. difficile* toxin B. Fourfold dilution range of pre-immune sera (day 0) and sera taken after third immunization (day 28) of llama 19 and 20 analyzed for binding to toxin B.

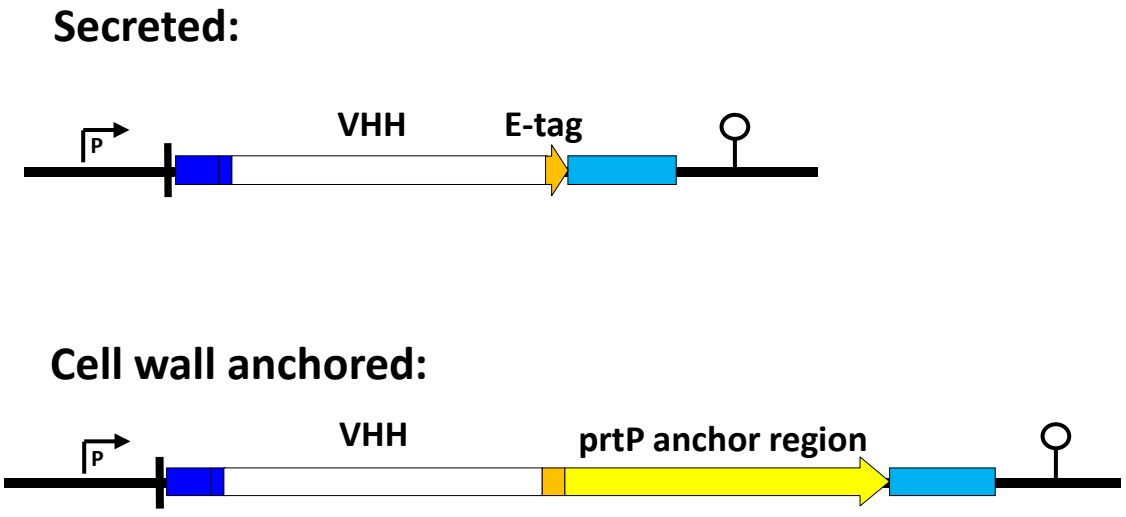

Figure S2. Plasmid constructs for expression of VHH fragments in *L. paracasei* BL23. The expression plasmids based on the expression cassette of the *apf* gene from *L. crispatus* M247. The inclusion of a PrtP anchoring domain and placement of the stop codon gives either secreted expression or cell wall anchoring of the VHH fragment. The APF promoter (P), APF signal peptide (blue), VHH (white), prtP anchor (yellow), E-tag (orange), translational stop codon (arrowhead), non-translated APF anchoring domain (light blue) and transcriptional terminator (lollipop) are indicated.

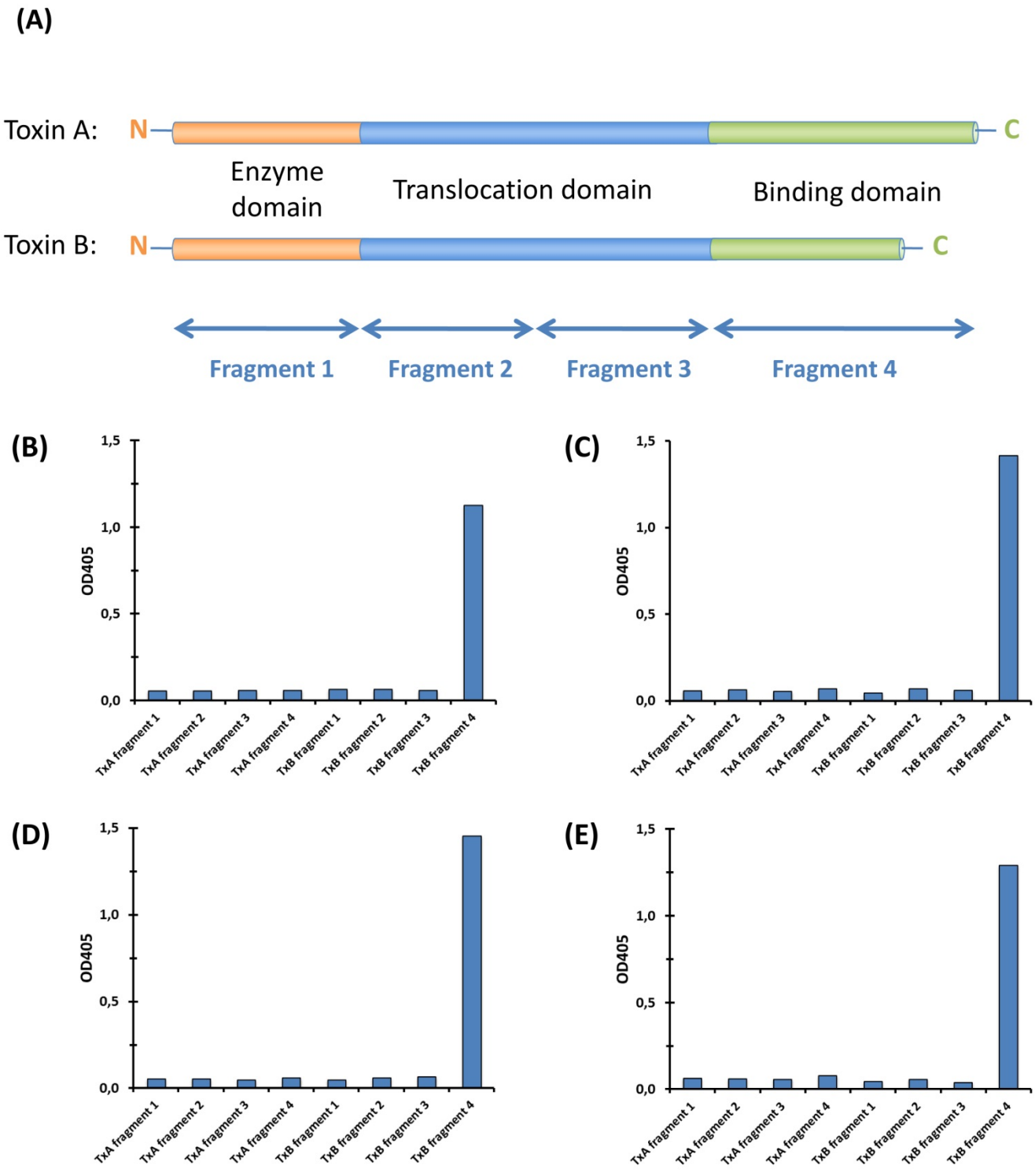

Figure S3. Mapping of domain specificity of *L. paracasei* BL23 produced anti-toxin B VHH on toxin A and toxin B. (A) Schematic representation of the toxin A and toxin B domains and the corresponding recombinant fragments produced in *E. coli*. (B) All tested VHH fragments (VHH-B2, VHH-E2, VHH-G3, VHH-D8) bind to the toxin B fragment 4, corresponding to the C-terminal receptor binding domain.

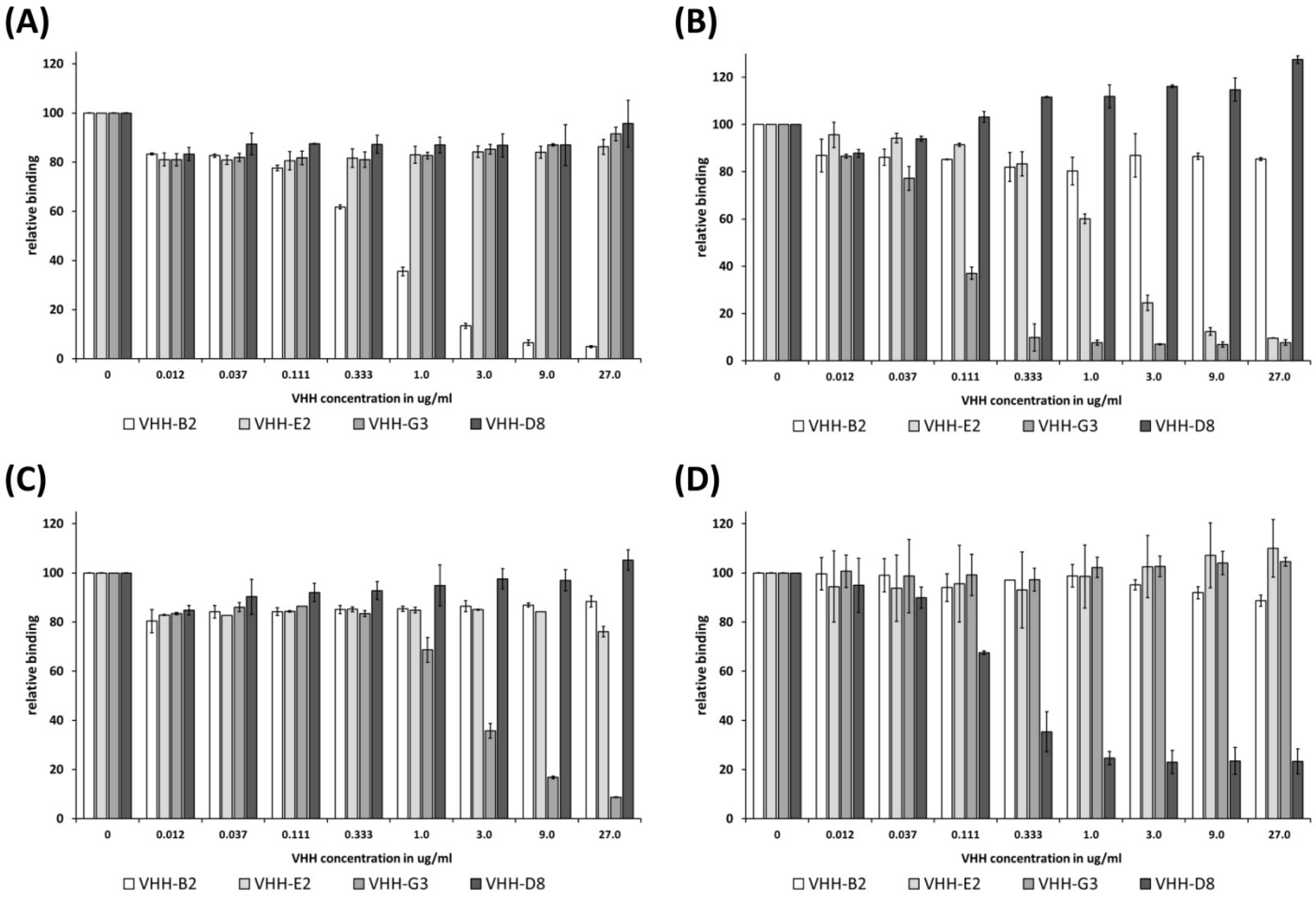

Figure S4. Epitope competition of toxin B neutralizing VHH fragments detected by ELISA. Binding of *Lactobacillus* produced VHH fragments to toxin B as coating antigen competed with purified *E. coli* produced VHH fragments in threefold dilutions. Binding was detected through the E-tag fused to the *Lactobacillus* produced VHH. (A) VHH-B2 produced by strain KKA442. (B) VHH-E2 produced by strain KKA440. (C) VHH-G3 produced by strain KKA382. (D) VHH-D8 produced by strain KKA441.

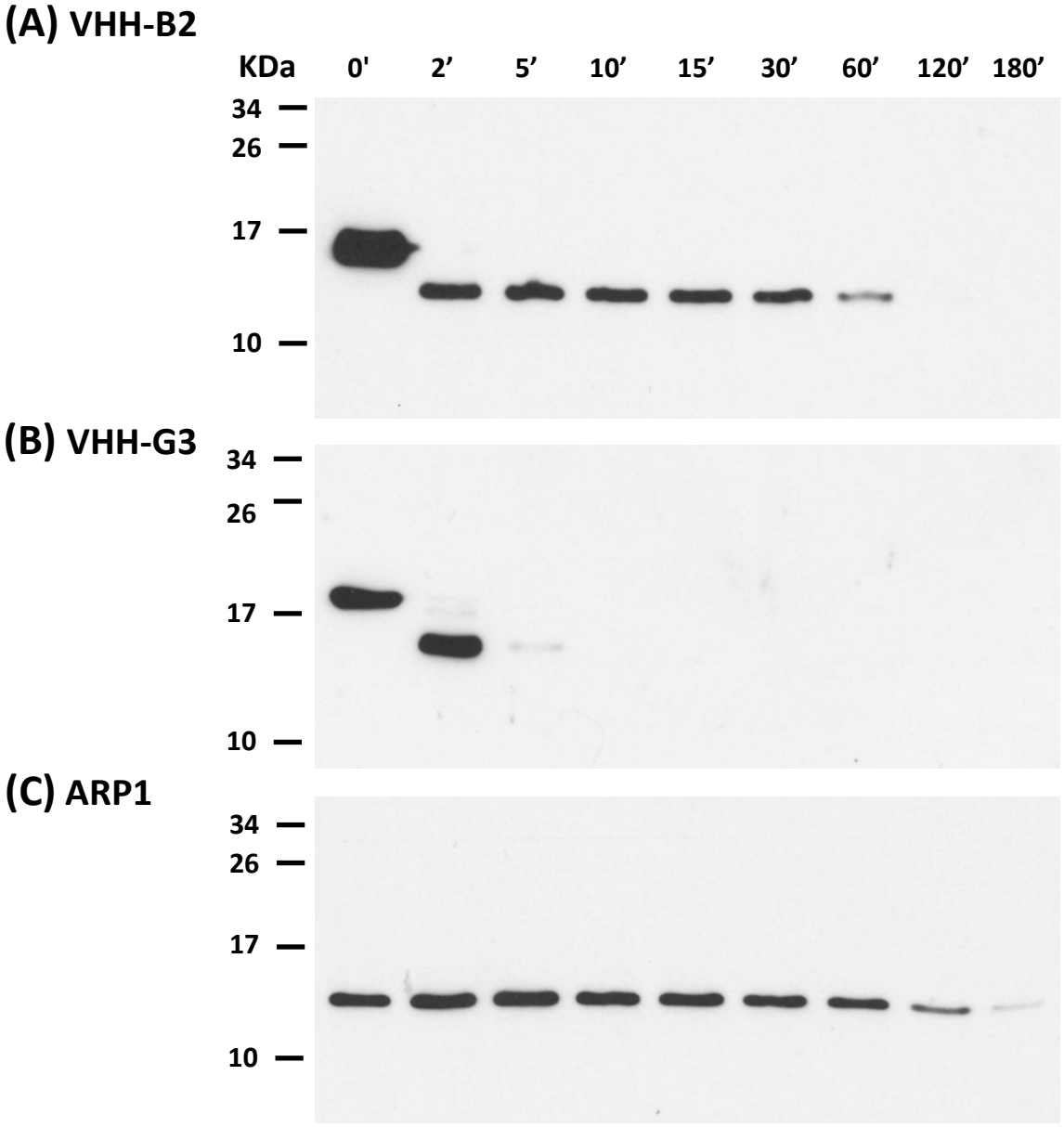

Figure S5. Proteolytic stability of toxin B neutralizing VHH fragments against intestinal proteases. Detection of degradation of VHH fragments exposed to hamster intestinal extracts by immunoblotting using an anti-VHH antibody. Western blotting of (A) anti-toxin B VHH-B2 and (B) anti-toxin B VHH-G3 exposed to hamster intestinal proteases for up to 180 minutes. (C) With anti-rotavirus VHH, ARP1 , as control for relative stability of the VHH fragments.