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1 **Bioactive immune components of anti-diarrheagenic Enterotoxigenic *E. coli* (ETEC)**
2 **hyperimmune bovine colostrum products**

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9 Running Head: Immune components of anti-diarrheal bovine colostrum

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20 **Abstract**

21 Diarrhea is a common illness among travelers to resource-limited countries, the most prevalent
22 attributable agent being enterotoxigenic *Escherichia coli* (ETEC). There are presently no
23 vaccines licensed specifically for the prevention of ETEC-induced traveler's diarrhea (TD), and
24 this has propelled investigation of alternative preventive methods. Colostrum, the first milk
25 expressed after birthing, is rich in immunoglobulins and innate immune components for
26 protection of newborns against infectious agents. Hyperimmune bovine colostrum (HBC)
27 produced by immunization of cows during gestation (and containing high levels of specific
28 antibodies) is a practical and effective prophylactic tool against gastrointestinal illnesses. A
29 commercial HBC product, Travelan®, is available for prevention of ETEC-induced diarrhea.
30 Despite its demonstrated clinical efficacy, the underlying immune components and anti-
31 microbial activity that contribute to protection remain undefined. We investigated innate and
32 adaptive immune components of several HBC formulated to reduce the risk of ETEC diarrhea,
33 including Travelan® and IMM-124E, a newer product that has broader gastrointestinal health
34 benefits. The immune components measured included total and ETEC-specific IgG, total IgA,
35 cytokines, growth factors, and lactoferrin. HBC products contained high levels of IgG specific for
36 multiple ETEC antigens, including O-polysaccharide 78 and Colonization Factor Antigen-1
37 (CFA/I) present in the administered vaccines. Anti-microbial activity was measured *in vitro* using
38 novel functional assays. HBC greatly reduced ETEC motility in soft agar and exhibited
39 bactericidal activity in the presence of complement. We have identified immune components
40 and antimicrobial activity potentially involved in the prevention of ETEC infection by HBC *in vivo*.

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44 **Introduction**

45 Enterotoxigenic *Escherichia coli* (ETEC) is a major etiology of bacterial diarrhea in
46 children living in resource-limited countries and the main attributable cause of diarrhea in
47 travelers and military personnel deployed to these regions (1-3). Usually self-limiting, classic
48 traveler's diarrhea (TD) is defined as three or more unformed bowel movements per 24 hours
49 often accompanied by at least one additional symptom such as stomach cramps, fever, nausea,
50 vomiting, and blood (1). In addition to being disruptive to travel, TD causes burdensome medical
51 expenses, loss of productivity, and important post-infectious sequelae including irritable bowel
52 syndrome (4, 5). The prevalence of ETEC infection and the morbidity associated with it
53 underscore the necessity for effective preventive tools.

54 There is no commercially available ETEC-specific vaccine. The licensed oral cholera
55 vaccine, Dukoral®, is recommended for a secondary indication to prevent TD caused by ETEC
56 that express heat labile enterotoxin (LT) due to the homology between the cholera toxin B (CTB)
57 and the ETEC LT B subunits. However, this vaccine has a modest protective efficacy
58 (approximately 50-60%) against ETEC-induced TD (6, 7), and while available in Europe and
59 Canada, Dukoral® is not licensed in the USA (8). Several whole cell and subunit ETEC vaccine
60 candidates have been investigated in preclinical and clinical studies with varying levels of
61 success (7, 9). Progress in developing a broadly protective ETEC vaccine has been hindered by
62 regional differences in diversity and prevalence of ETEC serotypes, colonization factors, and
63 other antigens (10) and our limited understanding of the immunological effectors required to
64 achieve protective immunity. LT along with the heat stable toxin (ST) contribute to the
65 extravasation of intestinal fluid and while ST is poorly immunogenic, LT neutralizing antibodies
66 contribute to protection from illness (11, 12). Antibodies that can block host-cell attachment
67 through binding to cell-surface fimbrial antigens (coli surface antigens [CS]) and colonization
68 factors (CF), including colonization factor antigen I (CFA/I), have been associated with reduction

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69 of symptomatic disease (13-16). However, the association between serum IgG titers and
70 protection does not apply to all CSs (14). Further, over 25 fimbrial antigens have been
71 characterized and more than one may be expressed by a particular isolate (17).

72 The challenges of ETEC vaccine development have spurred a search for alternative
73 prophylactic approaches. Foremost among these have been fortified natural compounds or
74 nutraceuticals, such as bovine colostrum, the nutrient-enriched milk produced within 24 hours of
75 birthing. Bovine colostrum contains high levels of antibodies, cytokines, growth factors, and
76 antimicrobial peptides and passively protects newborn calves from environmental pathogens
77 while their immune system develops (18-20). The therapeutic benefit of bovine colostrum to
78 human health has long been recognized. In fact, bovine colostrum concentrates have been
79 widely used as nutritional supplements and therapeutics against gastrointestinal (GI) pathogens
80 (21, 22). Hyperimmune bovine colostrum (HBC) with high concentrations of IgG for a specific
81 pathogen is produced by repeated immunization of pregnant cows. The use of HBC rich in
82 microbe-specific IgG for the prevention and treatment of GI infections has the advantages of
83 being a safe product (with standard dairy farming and manufacturing practices followed) that
84 humans consume regularly, and unlike antibiotics, they do not disturb the gut microbiome (23).

85 An early clinical study conducted by Tacket et al. showed that daily consumption of an
86 ETEC hyperimmune bovine milk concentrate shortly after each meal protected volunteers from
87 an experimental oral challenge with $1.2x10^9$ colony forming units (CFU) of ETEC virulent strain
88 H10407 (24). In a subsequent study by Otto and colleagues, an ETEC HBC delivered prior to
89 each meal reduced the incidence and volume of diarrheal stools in over 90% of volunteers orally
90 challenged with $1x10^9$ CFU H10407 (25). These HBC products were the precursors of
91 Travelan®, an anti-ETEC HBC manufactured by Immuron Ltd. and commercially available for
92 prophylaxis of TD in the USA, Australia, and Canada (http://www.immuron.com/). A similar
93 newer product produced by Immuron, designated IMM-124E, is being tested in humans to

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94 assess gastrointestinal and systemic (i.e. liver) therapeutic benefits (ClinicalTrials.gov Identifier
95 NCT02316717).

96 Apart from the demonstrated clinical efficacy of HBC in preventing enteric infections (22,
97 26), there is little information regarding the specific immune components and mechanisms that
98 mediate their immune modulatory and protective activity. Hence, the goal of this study was to
99 characterize the innate and adaptive immune components of HBC products that might
100 contribute to their health-improving effects and disease prevention. To this end, we investigated
101 the presence of cytokines, growth factors, lactoferrin, as well as total IgG and IgA and ETEC-
102 specific IgG in IMM-124E powders and tablets, alongside the commercial product Travelan® and
103 non-hyperimmune bovine milk control. Most importantly, we established *in vitro* functional
104 assays and examined the anti-microbial activity of HBC products, specifically their ability to limit
105 ETEC motility and promote bacterial killing in the presence of complement, as possible
106 mechanisms by which they limit ETEC disease in humans.
107

108 **Results**

109 **Innate immune components in bovine colostrum products (BCP).** We initially examined the
110 presence of non-specific innate immune components in BCP, including a subset of growth
111 factors, i.e. epidermal growth factor (EGF), growth hormone (GH), insulin growth factor 1 (IGF-
112 1), transforming growth factor-beta 2 (TGF-β2), tumor necrosis factor-alpha (TNF-α), interferon-
113 gamma (IFN-γ), as well as cytokines, i.e. interleukin (IL-) 1 beta (IL-1β), IL-2, IL-4 and IL-6, and
114 the antimicrobial protein lactoferrin. These components, reportedly present in unprocessed
115 bovine colostrum and milk, were first measured in whole, normal colostrum and in bovine serum
116 to confirm that they could be adequately quantified by the commercial assays used (Table 1). All
117 of the cytokines and growth factors tested, except IL-6 and TGF-β2, were detected in bovine
118 serum. GH, IGF-1, IL-1β, IL-4, TNF-α, and lactoferrin were detected in unprocessed colostrum.

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119 IL-6 and TGF-β2 were undetectable in colostrum or in serum, as were EGF, IFN-γ and IL-2
120 (Table 1).
121 We next investigated the presence of innate immune components in several HBC
122 powders (active ingredient) and tablets (final formulation for human consumption): 1) IMM-124E
123 1-9, representing 9 different lots of HBC powder, each derived from a group of Holstein cows
124 immunized with either a formalin-inactivated O78 serotype ETEC vaccine strain (1/3 of each
125 group) or with a mix of multiple formalin-inactivated ETEC strains of different serotypes (2/3 of
126 each group); 2) IMM-124E, a blend of the 9 individual powders IMM-124E 1-9; 3) IMM-124E
127 tablets, and 4) Travelan®. Normal cow milk powder and tablets (ProMilk) containing the same
128 excipient composition but lacking the HBC active ingredient were included as non-hyperimmune
129 controls. These samples are described in detail in Table 2. The biomarker composition was
130 examined comparing IMM-124E individual vs. blended powders (IMM-124E 1-9), IMM-124E
131 powder vs. tablets, and IMM-124E tablets vs. Travelan®.
132 GH, IGF-1, TNF-α were detected in all of the HBC products. The levels measured in the
133 IMM-124E 1-9 individual powders were similar to those of the IMM-124E blended powder (i.e.,
134 the latter falling within ± 2 standard deviations (SD) of the IMM-124E 1-9 mean value), and
135 above the ProMilk control (Figure 1 A-D). The IMM-124E powder and tablets had similar GH
136 content, while the powder had an excess of IGF-1 and TNF-α as compared with the tablets
137 (above the mean + 2 SD of amount measured in the tablets). All of the HBC products contained
138 large quantities of lactoferrin. The amount of lactoferrin in IMM-124E 1-9 was similar to that of
139 IMM-124E blended powder. An unexpected high level of lactoferrin, surpassing that of IMM-
140 124E, was found in the ProMilk powder but not in the ProMilk tablets (Figure 1 D). Importantly,
141 we found no statistically significant differences in the amounts of GH, IGF-1, TNF-α, and
142 lactoferrin when comparing IMM-124E and Travelan® tablets (Figure 1). None of the remaining
143 cytokines and growth factors tested (i.e. IFN-γ, IL-1β, IL-2, IL-4, IL-6, and TGF-β2) were

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144 detected in the HBC. EGF was found only in ProMilk and in two of the IMM-124E individual
145 powders, at levels ranging from 0.017-0.063 ng/mg of BCP.
146
147 **Total and ETEC-specific antibodies in BCP.** We subsequently determined the total IgG, IgG1,
148 IgG2, and IgA content in the BCP mentioned above (Figure 2). Total IgG and IgG1 were the
149 predominant antibody class and subclass in the HBC products (Figure 2 A and B), while IgG2
150 and IgA amounted to less than 10% of the total IgG in all of the samples tested (Figure 2 C and
151 D). The antibody levels (IgG, IgG1, IgG2, and IgA) in the IMM-124E 1-9 individual powders were
152 similar to those of the IMM-124E blended powder and above the levels found in ProMilk. They
153 were also similar between the IMM-124E blended powder and tablets. The levels of total IgG
154 and IgG1 in IMM-124E tablets were not significantly different from those of Travelan®. The
155 amounts of IgG2 and IgA in IMM-124E tablets appeared higher than those of Travelan®, yet the
156 differences were not statistically significant.
157 Given the similar IgG composition of the IMM-124E 1-9 individual and IMM-124E
158 blended powder, ETEC-specific IgG antibodies were measured only in the blended IMM-124E
159 HBC, as well as in the IMM-124E tablets, Travelan®, and ProMilk controls (Figure 3). IMM-124E
160 powder and tablets had comparable levels of ETEC O6 and O78 lipopolysaccharide (LPS)-
161 specific IgG that were above the mean level in the ProMilk controls (8- and 15-fold higher in
162 IMM-124E tablets compared to ProMilk tablets, respectively, Figure 3 A and B). IMM-124E
163 powder and tablets also contained CFA/I-, CFA/II-, CS3-, CS4-, and CS6-specific IgG; levels
164 were at least five times higher compared to ProMilk (Figure 3 C-G). In addition to antibodies
165 directed to cell surface antigens, the HBC products contained high levels of LT-specific IgG
166 (Figure 3 H). Of note, except for O78 and LT, ETEC-specific IgG titers in IMM-124E powder and
167 tablets surpassed those of Travelan®.
168 We next examined the presence of cross-reactive antibodies to *E. coli* LPS serotypes
169 not included in the vaccine (i.e. O42, O55, and O127) but known to be expressed in clinically

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170 relevant isolates. IgG recognizing these non-vaccine serotypes were detected in IMM-124E
171 powder and tablets at levels at least twice as high as those of ProMilk tablets (Figure 4). Cross-
172 reactive ETEC IgG titers were also similar between IMM-124E tablets and Travelan®.
173 We also queried the possibility of IMM-124E IgG antibodies exhibiting cross-reactive
174 binding to LPS from other Gram-negative enteric pathogens: *Shigella flexneri*, *sonnei,* and
175 *dysenteriae,* and *Salmonella enterica* serovars Typhi, Enteriditis, and Typhimurium. IgG-
176 recognizing *Shigella* and *Salmonella* LPS were detected in the IMM-124E powder and tablets,
177 as well as in Travelan®; levels were at least 4-fold as high as those of ProMilk (data not shown).
178
179 **Anti-microbial activity of BCP.** To assess the anti-microbial activity of BCP we developed a
180 functional assay that allowed determination of inhibition of ETEC motility. ETEC strain H10407
181 was selected as the main target to measure antibody functionality because it was the strain
182 used in multiple human challenge studies to assess vaccine and therapeutic efficacy (27). A
183 pool of sera from volunteers challenged with strain H10407 was used as positive (immune)
184 control, and normal (non-immune) serum as negative control. Motility of strain H10407 in soft
185 agar was significantly reduced in the presence of immune sera (Figure 5 A). Further, the
186 reduction of motility was proportional to the amount of immune sera added to the agar (Figure 5
187 A). Likewise, we assessed the capacity of the HBC supernatants (diluted 1:2 and embedded in
188 agar) to inhibit ETEC motility. IMM-124E powder and tablets, as well as Travelan®, significantly
189 inhibited H10407 motility compared to ProMilk (Figure 5 B). Similar inhibition of motility was
190 observed when testing the IMM-124E 1-9 individual powders (data not shown). The IMM-124E
191 tablets also significantly reduced the motility of strains that express other fimbrial and O
192 antigens present in the vaccines given to the cows, i.e. E11881A (O25:H42, CS4, CS6), E9034
193 (O8:H9, CS3, CS21), and E1392/75-2A (O6:H16, CFA/II) (Figure 5 C). Representative images
194 showing inhibition of ETEC motility in the presence of IMM-124E or ProMilk are shown in Figure

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195 5 D. The assay was performed in media that promotes expression of multiple ETEC virulence
196 factors (28).
197 A bactericidal assay was established to examine the capacity of HBC antibodies to
198 promote microbial killing in the presence of complement. Antibody-dependent complement-
199 mediated colostrum bactericidal activity (CBA) titers were determined as the inverse of the BCP
200 (reconstituted supernatant) dilution corresponding to 50% killing. Titers for the negative and
201 positive (H10407 immune) controls were <10 and >7,000, respectively. CBA activity was first
202 examined in the IMM124-E 1-9 individual powders; end-point titers ranged between 124 and
203 270 and greatly surpassed the titer of ProMilk control (mean <20). CBA titers were also
204 determined in the IMM-124E blended powder and tablets and levels were found to be similar
205 (407 and 369, respectively) and significantly higher than those of ProMilk (Figure 6 A). There
206 was no statistically significant difference between the CBA activity in IMM-124E and Travelan®.
207 We did not detect consistent dose-dependent killing when HBC was tested against E11881A,
208 E9034, and E1392/75-2A (data not shown). Given that antibodies specific for O-polysaccharide
209 antigens are known to be involved in complement-mediated killing against other Gram-negative
210 organisms, we attempted to determine their contribution in the observed ETEC killing. To this
211 end, CBA titers of IMM-124E powder and tablets (supernatants) were measured after
212 adsorption of O78 serotype-specific antibodies with increasing amounts of O78 capsular
213 polysaccharide. Bacterial killing, though variable, was significantly reduced when O78-specific
214 antibodies were sequestered, as observed for the H10407 (O78) positive control sera (Figure 6
215 B). Bactericidal activity of the IMM-124E powder and tablets were reduced, but not completely
216 abrogated by adsorption of O78-specific antibodies (Figure 6 B).
217
218 **Discussion**
219 Bovine colostrum is a safe and effective nutraceutical sought for the prevention and
220 treatment of a variety of infectious diseases and immune disorders (26). HBC rich in pathogen-

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221 specific immunoglobulins has been used prophylactically to prevent illness associated with
222 gastrointestinal infections, including ETEC diarrhea (23, 26). Two products manufactured by
223 Immuron Ltd. were the focus of our study: Travelan®, a commercially available anti-ETEC HBC
224 for prevention of TD, and IMM-124E, a more recent anti-ETEC HBC intended not only for
225 prevention of TD but also as a therapeutic to improve systemic chronic inflammatory conditions
226 such as metabolic syndrome and non-alcoholic steatohepatitis (NASH). Travelan® has been
227 shown to reduce both the incidence and severity of ETEC-induced diarrhea in up to 90% of
228 volunteers (24, 25), and a similar prophylactic activity is expected of IMM-124E. While the
229 efficacy of HBC is attributed mainly to the heightened concentration of pathogen-specific
230 antibodies, the exact immune composition of these products and the basis for their clinical
231 protection remain unknown. This study represents the first detailed characterization of immune
232 components and *in vitro* anti-microbial properties of HBC; the analysis included a comparison of
233 active ingredient and final formulation, as well as comparison of a new and an established
234 product with demonstrated clinical efficacy.

235 Our analysis of innate immune components revealed an abridged profile of cytokines
236 (TNF-α) in HBC products as compared to fresh unprocessed colostrum (IL-1β, IL-4, and TNF-
237 α). The difference might reflect the intrinsic composition of these products (HBC was produced
238 in New Zealand whereas the fresh colostrum was harvested in the US) and/or changes due to
239 processing. The loss of components during manufacturing can affect the products' potency and
240 consistency, and it is therefore important to document and understand its occurrence. Our
241 comparative analysis of IMM-124E active ingredient powder and tablets aimed precisely at
242 identifying changes in composition due to formulation processing. The comparable results
243 obtained for IMM-124E powder and tablets in every test performed confirmed the preservation
244 of active ingredients in the final product. The HBC preparations contained lactoferrin, a potent
245 anti-microbial protein capable of inhibiting ETEC growth and adherence to epithelial cells in vitro
246 (29, 30). Interestingly, higher levels of lactoferrin were found in ProMilk as compared with the

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247 HBC powders and tablets, possibly reflecting the concentrated proteins contained in this
248 product. Notwithstanding, ProMilk did not exhibit significant anti-microbial activity when
249 compared to the HBC products.

250 Aside from levels, the biological activity of the innate immune molecules detected in the
251 HBC remains to be determined. Because of their complexity, the biological effects of milk-
252 derived products have largely been explored using whole preparations, rather than teasing apart
253 the role of specific molecules. Fresh bovine colostrum and milk are known to promote growth of
254 human intestinal cells (31) and a spray-dried colostrum product was shown to prevent gut
255 dysfunction and inflammation in pre-term pigs (32). We anticipate the HBC products examined
256 would exhibit similar intestinal health restoring properties. Oral administration of IMM-124E was
257 found to reduce local and systemic inflammation and promote peripheral Tregs in clinical
258 studies (http://immuron.com/assets/Uploads/AASLD-Poster-2010-NASH-clinical-trial-final.pdf).

259 The HBC in this study was designed to prevent ETEC diarrhea, hence the demonstration
260 of pathogen-specific adaptive immunity was particularly relevant. The high levels of antibodies
261 against the target vaccine antigens O6 and O78 polysaccharides, CFA/I, CFA/II, CS3, CS4,
262 CS6, and LT detected in IMM-124E and Travelan® reflect the purposeful hyperimmunization.
263 These antibodies are believed to provide the basis for the clinical protection afforded by HBC in
264 ETEC challenged volunteers, which included reduced rate of illness and abdominal pain (24,
265 25). HBC antibodies also recognized ETEC O-serotypes not included in the vaccines including
266 O44, O55, and O127, as well as *Shigella* and *Salmonella* O-polysaccharides, likely due to the
267 presence of shared epitopes. Furthermore, LT has homology with CT and antibodies directed to
268 either toxin are known to be cross-reactive. It is therefore plausible that the HBC studied might
269 have a beneficial effect in preventing infections by other (non-ETEC) enteric pathogens.

270 An important contribution of this study is the demonstration of functional anti-microbial
271 activity of HBC products, attributed mainly to ETEC-specific antibodies. We are the first to report
272 the development of assays to determine inhibition of ETEC motility and antibody-mediated

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273 complement-dependent bacterial killing, and to demonstrate the presence of functional
274 antibodies in human ETEC-immune sera and anti-ETEC HBC. Antibody activity, as opposed to
275 antibody levels, more faithfully reflects the capacity of antibodies to block organisms and
276 promote their clearance *in vivo*. Functional assays are also valuable for confirming the biological
277 competency of the HBC active ingredients and may be useful quality control tools for the
278 manufacturing process. The anti-microbial activity for different lots of the same HBC (IMM-124E
279 and Travelan®) was noticeably different, which emphasizes the importance of individual lot
280 analysis and confirms the suitability of the assay to discriminate biological activity of different
281 preparations.

282 We investigated antibody-mediated inhibition of motility because, along with adherence,
283 it is a key step of ETEC pathogenesis (33). Antibodies specific for flagellin and fimbriae are
284 likely responsible for inhibiting ETEC motility, although antibodies to CFA/I may also contribute
285 to abrogating motility (34). Antibodies to LPS, which reportedly inhibited *V. cholerae* motility
286 (35) may also play a role. Inhibition of ETEC motility by IMM-124E tablets and Travelan® varied
287 depending on the strain, with higher percent inhibition detected against strain H10407. Such
288 enhanced activity likely reflects the vaccine composition (1/3 of the herd received exclusively
289 formalin-inactivated H10407, while the remainder received a mix of other strains) or antigenic
290 dominance, which ultimately resulted in an abundance of H10407-specific antibodies.

291 IMM-124E and Travelan® also promoted killing of H10407 in the presence of
292 complement. The O-polysaccharide is a known target for bactericidal antibodies (36, 37). We
293 confirmed that this is also true for ETEC, as bacterial killing activity of immune serum and HBC
294 significantly declined commensurate with serotype-specific antibody adsorption of O78
295 polysaccharide antigen prior to incubation with bacteria and complement. The fact that killing
296 could not be completely abrogated, even for the O78-specific human sera and using larger
297 amounts of O78, suggests that additional antigens might also be targets for antibody-mediated
298 killing. The analysis of specificity of the functional antibody activity deserves further study.

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299 Serum bactericidal antibody levels have been associated with clinical protection against
300 diarrhea and illness caused by other enteric bacteria such as *Shigella* and *V. cholera* (36, 38,
301 39). It would be important to determine whether the inhibition of ETEC motility and bactericidal
302 activity observed is associated with disease prevention *in vivo*. Another important question is
303 whether these antimicrobial functions actually represent the mechanisms of protection. Antibody
304 binding to surface antigens may be sufficient to interfere with motility and attachment to host
305 receptors on the gut epithelium. The extent to which bovine antibodies effectively activate
306 human complement in the intestinal lumen is unknown. The observed lack of colostrum killing
307 activity of strains other than H10407 may be due to the relative amount of antibodies or
308 differences in strain biology, in particular the accessibility of antigens, which could also affect
309 reduction of motility. LPS chain length is known to affect *E. coli* sensitivity to complement-
310 mediated killing (40, 41). Similarly, serogroup-specific differences have been documented in
311 antibody and complement mediated killing of *Neisseria meningitidis* (42).

312 In summary, HBC used for prevention of ETEC diarrhea contains cytokines, growth
313 factors and lactoferrin that provide innate immune defenses and promote intestinal tissue
314 growth and repair. In addition, HBC contains high levels of ETEC-specific antibodies, primarily
315 IgG, including antibodies to key virulence factors. These antibodies have the capacity to inhibit
316 ETEC motility and promote complement-mediated lysis *in vitro*. These findings provide insights
317 into HBC innate immune components and antibody-mediated anti-microbial activities that help
318 prevent TD. The functional antibody assays developed will be useful in monitoring immunity
319 following infection and vaccination. These assays can also be tools to ensure quality of HBC
320 and antibody-based immunotherapies. Because of their safety profile, demonstrated biological
321 activity and clinical efficacy, anti-ETEC HBC represents a unique, natural, and efficacious
322 product to prevent TD.

323

324 **Materials and Methods**

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325 **Cow immunization and colostrum samples.** Immuron maintains a herd of approximately
326 7000 Holstein-Friesian cows. Pregnant cows were immunized with either a single-strain of *E.*
327 *coli* or a multi-strain *E. coli* vaccine before calving; approximately one third of the cows received
328 the single-strain vaccine while the remaining two-thirds received the multi-strain vaccine. The
329 single strain vaccine consisted of formalin inactivated extracts of an *E. coli* serotype O78 strain
330 (H10407) and the multi-strain vaccine consisted of formalin inactivated extracts of multiple *E.*
331 *coli* strains (and serotypes) including B2C (O6), C55 3/3c3 (O8), PE 595 (O15), E11881A
332 (O25), C1064-77 (O27), PE 672 (O63), E20738/0 (O114), PE 724 (O115), EI 37-2 (O128), B7A
333 (O148), E8772/0 (O153), and PE 768 (O159). The single strain vaccine was administered
334 intramuscularly in three 1ml doses, each containing 10^8 vaccine particles. The multi-strain
335 vaccine was delivered subcutaneously in three 1ml doses, each containing 10^8 vaccine particles
336 per strain. The vaccines were given 9 to 12 weeks before calving and then at 6 and 4 weeks
337 before calving. The immunization protocol was approved by the Office of the Chief Veterinary
338 Officer, Victoria, Australia, and conformed to the Australian Code of Practice for the Care and
339 Use of Animals for Scientific Purposes.

340 Colostrum from multiple milkings was collected within 24 h of calving from animals that
341 were immunized with either the single strain or multi-strain vaccines. All colostrum samples
342 were pooled and pasteurized with a single heat treatment of at least 72°C for a minimum of 15
343 sec. Fat and cream were removed prior to spray drying to produce the powders. The samples
344 investigated included (listed in Table 2): 1) commercial bovine serum from New Zealand cows
345 (ThermoFisher Scientific, Asheville, NC, USA); 2) unprocessed bovine colostrum obtained from
346 Holstein cows at Cornell University Department of Animal Science less than 24 h post-calving;
347 3) IMM-124E 1-9 individual powders, each representing mixed colostrum from different groups
348 of vaccinated cows; 4) IMM-124E, a blend of all 9 individual powders; 5) IMM-124E tablets (lots
349 D8925 and D9718), 4) Travelan® tablets (lots 47058 and 17135); 6) ProMilk 85 100-1, a

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350 commercial powdered milk concentrate (Tatura Milk Industries Ltd., Victoria, Australia) and 7)
351 ProMilk tablets (lots D8926 and D9719).
352
353 **Colostrum preparation.** For ELISAs, bovine colostrum and milk powders were dissolved in
354 PBS-Tween pH 7.4 (to a final concentration of 16 mg/ml), with shaking at room temperature for
355 2 h, and supernatants collected after centrifugation at 4000xg for 10 min. For functional assays,
356 BCP were dissolved in PBS pH 7.4 (to a final concentration of 64 mg/ml) and supernatants
357 obtained as described above. Samples were stored at 4°C and were used within two weeks of
358 preparation.
359
360 **Bacterial antigens and strains.** CFA/I, CFA/II, CS4, O78 core O-polysaccharide (COPS) were
361 purified from recombinant *E. coli* strains at the CVD (43, 44). *S. dysenteriae* LPS was also
362 produced at the CVD from strain CVD 1251 (45). The remaining antigens were obtained from
363 commercial vendors: CS3 and CS6 (BEI Resources, Manassas, VA, USA), LT (Lot #1735,
364 Berna Biotech, Switzerland), O6, O42, O55 and O127 LPS (Sigma-Aldrich, St. Louis, MO, USA)
365 *Salmonella* Enteriditis and Typhimurium LPS (Sigma-Aldrich), *S.* Typhi LPS (Becton Dickinson-
366 Difco, Franklin Lakes, NJ, USA), *S. flexneri* 2a strain 2457T LPS (WRAIR) , and *S. sonnei*
367 strain 53G LPS (WRAIR). ETEC strains H10407 (O78:H11, LT^+, ST^+, CFA/I), E1392/75-2A
368 (O6:H16, LT^-, ST^-, CFA/II), and E9034 (O8:H9, LT^+, CS3, CS21) and E11881A (O25:H42, LT^+,
369 ST^+, CS4, CS6) were obtained from CVD collections. Strains were maintained on tryptic soy
370 agar (TSA) and grown CFA media (containing 1% [w/v] casamino acids, 0.15% [w/v] yeast
371 extract, 0.005% [w/v] $MgSO_4$, 0.0005% [w/v] $MnCl_2$) at indicated times for functional antibody
372 assays.
373
374 **Measurement of antibodies.** Total IgG, IgG1, IgG2, and IgA titers were measured using ELISA
375 kits (Bethyl Laboratories, Montgomery, TX, USA). Single dilutions of each sample were tested

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376 in duplicate, and endpoint titers calculated by extrapolation on standard curves of known
377 immunoglobulin concentrations. Colostrum IgG antibodies specific for the following ETEC,
378 *Salmonella*, and *Shigella* antigens were measured by ELISA using the indicated coating
379 concentrations: CFA/I (7 µg/ml), CFA/II (5 µg/ml), CS3 (1 µg/ml), CS4 (1 µg/ml), CS6 (1 µg/ml),
380 LT (1 µg/ml), O6 LPS (5 µg/ml), O42 LPS (5 µg/ml), O55 LPS (5 µg/ml), O78 COPS (2.5
381 µg/ml), O127 (5 µg/ml), *Salmonella* Enteriditis LPS (5 µg/ml), *S.* Typhimurium LPS (2 µg/ml), *S.*
382 Typhi LPS (10 µg/ml), *Shigella dysenteriae* LPS (5 µg/ml), *S. flexneri* 2a strain 2457T LPS (5
383 µg/ml), and *S. sonnei* strain 53G LPS (5 µg/ml). A pool of sera from volunteers challenged with
384 the ETEC strain H10407 was used as positive (immune) control, and normal (non-immune)
385 serum as negative control (prepared from archived specimens collected during CVD study *E.*
386 *coli* CVD-13002, unpublished data) for CFA/I and O78. Positive (immune) and negative (non-
387 immune) serum pools were prepared from archived specimens collected during various CVD
388 clinical and pre-clinical studies.

389 CF/CS diluted in PBS, pH 7.4 was added to Immulon 2HB 96-well plates (ThermoFisher
390 Scientific) while LPS and COPS diluted in carbonate-bicarbonate buffer, pH 9.6 were added to
391 medium binding microplates (Grenier Bio-one, Monroe, NC, USA). Plates were coated for 3 h,
392 washed and blocked overnight at 4°C (CF/CS antigens) or for 2 h at 37°C (LPS antigens). Wells
393 used to test bovine samples were blocked with 5% w/v casein-PBS buffer and wells used for
394 human controls were blocked 10% (w/v) non-fat dry skim milk (NFDM, [Nestle, Salon, OH,
395 USA]). HBC suspensions were diluted in PBS-Tween (0.1% [v/v]) with casein (0.5% [w/v])
396 (Sigma-Aldrich), and human sera control pools were diluted in PBS-Tween (0.05% [v/v] Tween
397 in PBS) containing 10% (w/v) NFDM; samples were added to the plates and incubated for 1 h at
398 37°C. Specific antibodies were detected using horseradish peroxidase (HRP)-labeled goat anti-
399 bovine IgG antibody diluted at 1:500 (KPL, Kirkegaard & Perry Laboratories, Inc., Gaithersburg,
400 MD, USA) in PBS-Tween with 0.5% (w/v) casein or HRP-labeled goat anti-Human IgG antibody
401 diluted at 1:10,000 (Jackson ImmunoResearch, West Grove, PA, USA) in PBS-Tween with 10%

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402 (w/v) NFDM followed by TMB Microwell Peroxidase substrate (KPL). Absorbance values at 450
403 nm were measured on a microplate spectrophotometer (Multiskan FC Ascent, Life Technologies
404 Corp., Carlsbad, CA, USA). End-point titers (EU/ml for antigen-specific antibodies) were
405 calculated as the inverse of the serum dilutions that produced an absorbance value at 450 nm
406 (A_{450}) of 0.2 above the blank. Antibody levels are reported as ELISA units (EU) per mg Total
407 IgG.
408

409 **Measurement of cytokines.** Epidermal Growth Factor (EGF) (CUSABIO Biotech, Hubei,
410 People's Republic of China [PRC]); Lactoferrin (Bethyl Laboratories); Growth Hormone (GH),
411 Interferon gamma (IFN-γ), Interleukin (IL) 1 beta (IL-1β), IL-2, IL-4, IL-6, Insulin-like Growth
412 Factor (IGF-1), Transforming Growth Factor beta 2 (TGF-β2), and Tumor Necrosis Factor alpha
413 (TNF-α) (Cloud-Clone Corp., Hubei, PRC) were measured using ELISA kits following
414 manufacturers' instructions. A_{450} values were measured on a microplate spectrophotometer as
415 described above with the exception of IFN-γ and TGF-β2 determinations, for which
416 luminescence signals were read on a luminometer (Tecan, Switzerland) at 1 second per well.
417 Samples were tested undiluted, in duplicate, and endpoint titers were calculated by the
418 extrapolation of A_{450} values on standard curves of known concentrations of the respective
419 analytes. Results are reported in mass per mg of reconstituted colostrum powder.
420

421 **Motility assay.** Colostrum powder supernatants were mixed 1:5 with CFA media in a 24-well
422 plate, eight replicates per sample. An equal volume of CFA media with 0.6% (w/v) agar and 1%
423 (w/v) 2,3,5-Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) was added to each well to yield
424 a final colostrum concentration of 6.4 mg/ml in 0.3% (w/v) agar. To prepare the assay strains,
425 overnight cultures were grown in Tryptic Soy broth (TSB) then diluted 1:100 in CFA media and
426 incubated at 37°C with shaking at 115 rpm until an OD_{600} of 0.7 to 1.0 was attained. A 1 µl drop
427 (7-9 x10^{8} cfu/ml) of culture was delivered just under the surface of the agar and in the center of

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428 each well containing the colostrum-agar mix; plates were incubated in a humidifying chamber at
429 37°C for 4-6 h. Images of each plate were obtained on a Chemi Doc MP Imaging System (Bio
430 Rad, Hercules, CA, USA). Only wells with circular or near circular areas of growth were included
431 for analysis. The diameter of bacterial growth in each well was determined using ImageJ
432 Software (NIH). The distance for zones of motility of hyperimmune colostrum products were
433 calculated and expressed as a percent of motility with respect to motility in ProMilk. Percent
434 inhibition of motility was calculated as the difference in growth between the mean of the
435 negative control (ProMilk) and the HBC, divided by the mean growth in the negative control
436 multiplied by 100. The mean of a minimum of four replicates was reported as the percent
437 inhibition of motility. Experiments were repeated at least three times with fresh milk or colostrum
438 suspensions.
439
440 **Complement-mediated Colostrum Bactericidal Activity Assay.** This assay was adapted
441 from the basic serum bactericidal assay format described by Boyd *et al* (36). Briefly, colostrum
442 powder suspensions were diluted in normal saline (0.85% sodium chloride) at 1:10 for non-
443 hyperimmune samples and 1:25 for hyperimmune samples, 100 µl was added to row 'A' of a 96-
444 well plate and then serially diluted two-fold in saline (for a total of 6 dilutions). The same pool of
445 human sera used for ELISAs were used as positive and negative controls diluted at 1:10 in
446 saline. Samples were assayed in duplicate. Overnight cultures of *E. coli* H10407, E11881A,
447 E1392/75-2A, and E9034 grown in TSB were diluted 1:1000 in CFA media and incubated at
448 37°C with shaking at 115 rpm until an OD_{600} of 0.1 to 0.2 was attained; bacteria were serially
449 diluted in PBS for viable counts on Tryptic Soy agar. Baby rabbit complement (BRC; Pel-Freez
450 Biologicals, Rogers, AK, USA) was diluted in normal saline (5 parts BRC to 3 parts saline) and
451 40 µl of the complement mix was added to each well followed by 10 µl of a 10^{-4} dilution of the
452 bacterial suspension in PBS. Each well had a final concentration of 15% BRC and
453 approximately 40 CFU of *E. coli*. Wells were mixed briefly by pipetting and plates were

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454 incubated at 37°C with shaking at 115 rpm for 1 hour. For viable counts, 2 x 10 µl drops per
455 well were plated on TSA plates and spread evenly by rocking; plates were incubated at room
456 temperature overnight. Colonies were counted and the percent killing calculated as (1 – mean
457 colony count per dilution) / (mean colony count of complement only control). Titers were
458 reported as the dilution of colostrum at which 50% of bacteria were killed, determined using the
459 Reed-Muench regression (46). Experiments were repeated at least three times with ProMilk or
460 colostrum suspensions.

461 The contribution of O78 O-polysaccharide to bactericidal activity was assessed by pre-
462 incubating colostrum suspensions diluted 1:25 with 12.5 µg/ml, 25 µg/ml, and 50 µg/ml O78
463 COPS for 30 min at 37°C with shaking at 115 rpm. Strain H10407 and BRC were then added
464 and the reaction completed as described above for standard CBA. Viable counts were recorded
465 and the percent bactericidal activity calculated as (1 – mean colony count per dilution) / (mean
466 colony count of complement only control).

467

468 **Statistical Analysis.** When values of an assay were available for replicates of a sample, the
469 median was taken as the value for the sample. The mean levels ± 2 SD for cytokines, growth
470 factors, and antibody were determined for IMM-124E powders 1-9 and considered as the range
471 of reference for these analytes in the HBC products. The mean levels ± 2 SD were compared
472 with the levels measured in the IMM-124E blended powder and ProMilk; a value for IMM-124E
473 blended powder or ProMilk was considered similar to the mean for IMM-124E powders 1-9 if it
474 was within the ± 2 SD range. In addition, IMM-124E blended powder was considered similar to
475 IMM-124E tablets when the value for the powder was within ± 2 SD of the mean for the tablets.
476 When more than one sample was available for comparison (e.g., for two lots of IMM-124E,
477 Travelan®, and/or ProMilk tablets), materials were compared using a two-sample t-test. When
478 more than one independent experiment was done, as for inhibition of motility and CBA titers,
479 IMM-124E and ProMilk powders were compared by paired t-test (one pair for each experiment)

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480 or one-sided paired t-test within each experiment (when multiple strains were included), and
481 Fisher's combined probability test, in which the p-values for each experiment were combined to
482 provide a chi-square test. IMM-124E tablets and Travelan® tablets were compared to ProMilk
483 tablets by one-sided two-sample t-test and Fisher's combined probability test. Two-sample t-
484 tests allowed for unequal variances. Statistical analyses were done using Microsoft Excel and
485 NCSS 10 software (Number Cruncher Statistical Systems, Kaysville, UT, USA). A two-sided p-
486 value < 0.05 was considered statistically significant. No adjustment was made for multiple
487 comparisons.

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494

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645 **Figure Legends**

646 **Figure 1.** Innate immune components in bovine colostrum products (BCP). (A) Growth hormone
647 (GH), (B) insulin growth factor 1 (IGF-1), (C) tumor necrosis factor-alpha (TNF-α) and (D)
648 lactoferrin were measured by ELISA in BCP resuspended in PBS-Tween (at a final
649 concentration of 16 mg/ml) as described in materials and methods. Samples tested included
650 ProMilk powders and tablets, IMM-124E 1-9 individual powders, IMM-124E powder and tablets,
651 and Travelan® (Table 2). Data represent the mean of duplicates when a single lot was tested
652 (ProMilk or blended IMM-124E powder) or mean + SD when multiple lots were available (IMM-
653 124E HBC 1-9, and 2 batches each of ProMilk, IMM-124E, and Travelan® tablets), as listed in
654 Table 2. Asterisks indicate statistically significant differences between HBC and ProMilk tablets
655 by two-sample t-test (* two-sided $p<0.05$).

656

657 **Figure 2.** Immunoglobulin composition of BCP. (A) Total IgG, (B) IgG1, (C) IgG2, and (D) IgA
658 were measured by ELISA as described in materials and methods. Samples tested included
659 ProMilk and IMM-124E, and Travelan® (Table 2). Data represent Ig class or subclass content as
660 mean of duplicates for single lot samples or mean of multiple lots + SD (where applicable).

661

662 **Figure 3.** Antibody titers specific to ETEC vaccine antigens in BCP. Specific IgG-antibodies to
663 vaccine antigens: (A) O6 LPS, (B) O78 COPS, (C) CFA/I, (D) CFA/II, (E) CS3, (F) CS4, (G)
664 CS6, and *E. coli* LT were measured by ELISA in ProMilk, IMM-124E, and Travelan®. Data
665 represent mean ELISA units (EU) (+SD when multiple lots were tested) per milligram of total
666 IgG. Asterisks indicate significant differences between HBC and ProMilk tablets by two-sample
667 t-test (* two-sided $p<0.05$).

668

669 **Figure 4.** Antibody titers specific to non-vaccine ETEC antigens in BCP. Specific IgG-antibodies
670 to ETEC antigens not included in the vaccine administered to the cows: (A) O42 LPS, (B) O55

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671 LPS, and (C) O127 LPS were measured by ELISA in ProMilk, IMM-124E, and Travelan®
672 described in Figure 1. Data represent mean ELISA units (EU) (+SD where applicable) of
673 antigen-specific IgG per milligram of total IgG. Asterisks indicate statistically significant
674 differences between HBC and ProMilk tablets by two-sample t-test (* two-sided p<0.05, ***
675 p<0.01).
676
677 **Figure 5.** BCP inhibition of ETEC motility. Bacteria were seeded at the center of wells
678 containing CFA agar mixed with serum or BCP. Migration was determined as the distance of
679 growth after a 4-6 h incubation and percent inhibition of motility was calculated with respect to
680 migration in no serum or ProMilk. (A)The motility assay was optimized with immune sera (at the
681 indicated dilutions) from volunteers challenged with strain H10407. Asterisks indicate significant
682 differences between inhibition of motility in the presence and absence of serum. (B-D) ProMilk,
683 IMM-124E, and Travelan® were tested for their capacity to inhibit ETEC motility. (B) Inhibition of
684 strain H10407 motility and (C) inhibition of strains E11881A, E1392/75/2A, and E9034 motility.
685 Data shown in A-C represent mean percent inhibition of motility + SD from at least three
686 experiments; asterisks indicate significant differences between HBC and ProMilk (*two-sided
687 p<0.05, **p<0.01, *** p<0.001). (D) Representative images of zones of motility (white bars) in
688 agar containing ProMilk and IMM-124E.
689
690 **Figure 6.** Complement-mediated colostrum bactericidal activity of BCP. ProMilk, IMM-124E,
691 and Travelan® were tested for the ability to mediate killing of strain H10407 in the presence of
692 complement. (A) Serially diluted ProMilk, IMM-124E, and Travelan® supernatants were
693 incubated with H10407 in the presence of baby rabbit complement for 1 h at 37°C and the
694 remaining viable organisms were quantified. End point titers were determined as the dilution at
695 which 50% of bacteria were killed using Reed-Meunch regression. Data represent mean
696 bactericidal titer + SD from three experiments. Asterisks indicate significant differences between

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697 HBC and ProMilk by paired t-test or Fisher's combined probability test (*** p<0.001). (B)
698 Inhibition of bactericidal activity by ETEC O78. Increasing concentrations of O78 COPS were
699 pre-incubated with BCP (diluted at 1:25) to deplete O78-specific antibodies and the complement
700 killing assay performed as described above. Data represent the percent bacteria killed with
701 respect to bacteria and complement-only control from three experiments. Asterisks indicate
702 significant differences between IMM-124E powder and tablets and ProMilk powder by Fisher's
703 combined probability test (*** p<0.001).
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708 TABLE 1. Concentration of immune components in normal, unprocessed, bovine colostrum and

709 serum

Component		Unprocessed Colostrum	Serum
Growth Factors	EGF	ND	0.5 ng/ml
	GH	9.4 ng/ml	7.5 ng/ml
	IGF-1	428.1 pg/ml	404.1 pg/ml
	TGFβ-2	ND	ND
Antimicrobial protein	Lactoferrin	1.6 ng/ml	0.4 ng/ml
Cytokines	IFN-γ	ND	191.2 pg/ml
	IL-1-β	48.2 pg/ml	45.9 pg/ml
	IL-2	ND	284.9 pg/ml
	IL-4	87.5 pg/ml	166.9 pg/ml
	IL-6	ND	ND
	TNF-α	398.6 pg/ml	133.6 pg/ml
Ig class/subclass	IgG	30.6 mg/ml	15.9 mg/ml
	IgG1	7.7 mg/ml	not tested
	IgG2	0.3 mg/ml	not tested
	IgA	5.9 mg/ml	not tested

710 ND=below detectable levels

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714 TABLE 2. Bovine milk and colostrum products used in this study

Sample type	Sample ID (abbreviation)	Description (Source)
Experimental colostrum powders	IMM-124E HBC 1 – 9	9 different lots of colostrum prepared from Holstein cow cohorts hyper-immunized with ETEC single strain or multi-strain vaccines (Immuron Limited)
	IMM-124E	A blend of IMM-124E HBCs lots1-9 (Immuron)
Control milk powders	ProMilk	A milk powder containing 85% milk proteins (Tatura Milk Industries Ltd., Australia)
Experimental colostrum tablets	IMM-124E, Lots D8925 and D9718	Composed of IMM-124E blended powder, 600 mg/tablet, (Immuron)
	Travelan®, Lots 47058 and 17135	Product for the reduction of risk of travelers' diarrhea and symptoms of minor GI disorders. Commercially available precursors to the HBC tablets made with IMM-124E DS (Immuron)
Control milk tablets	ProMilk, Lots D8926 and D9719.	Composed of ProMilk, 600 mg/tablet (Immuron)

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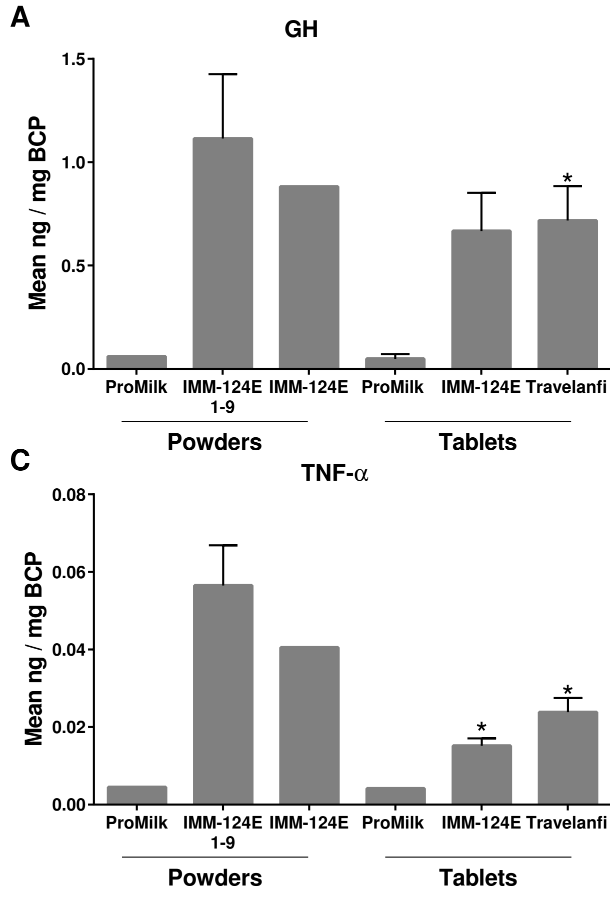

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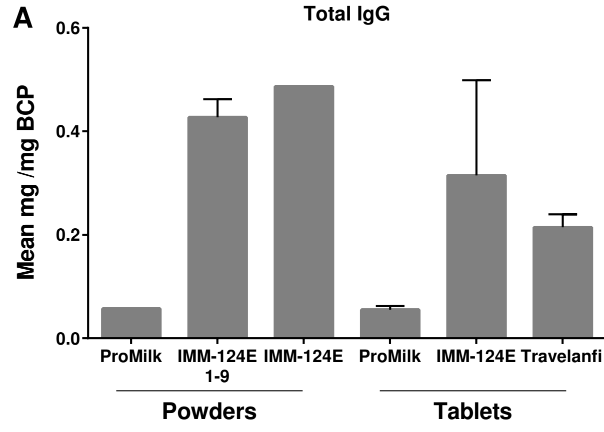

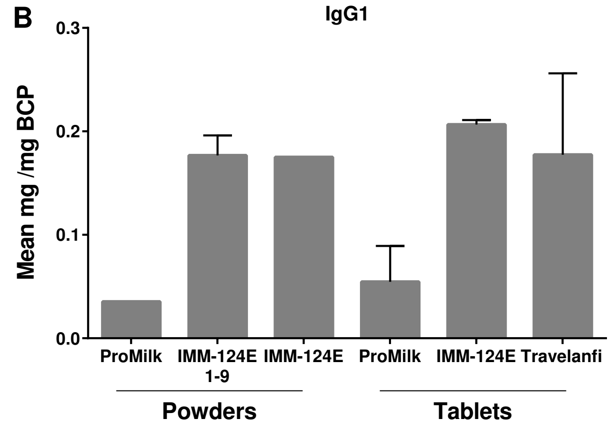

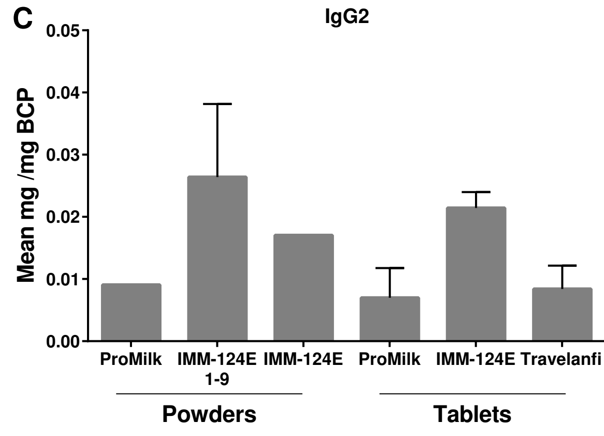

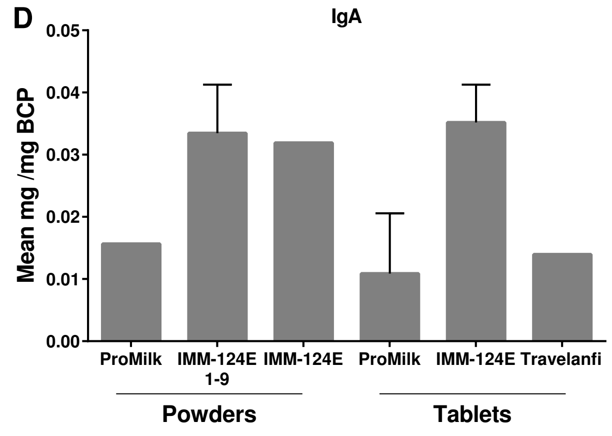

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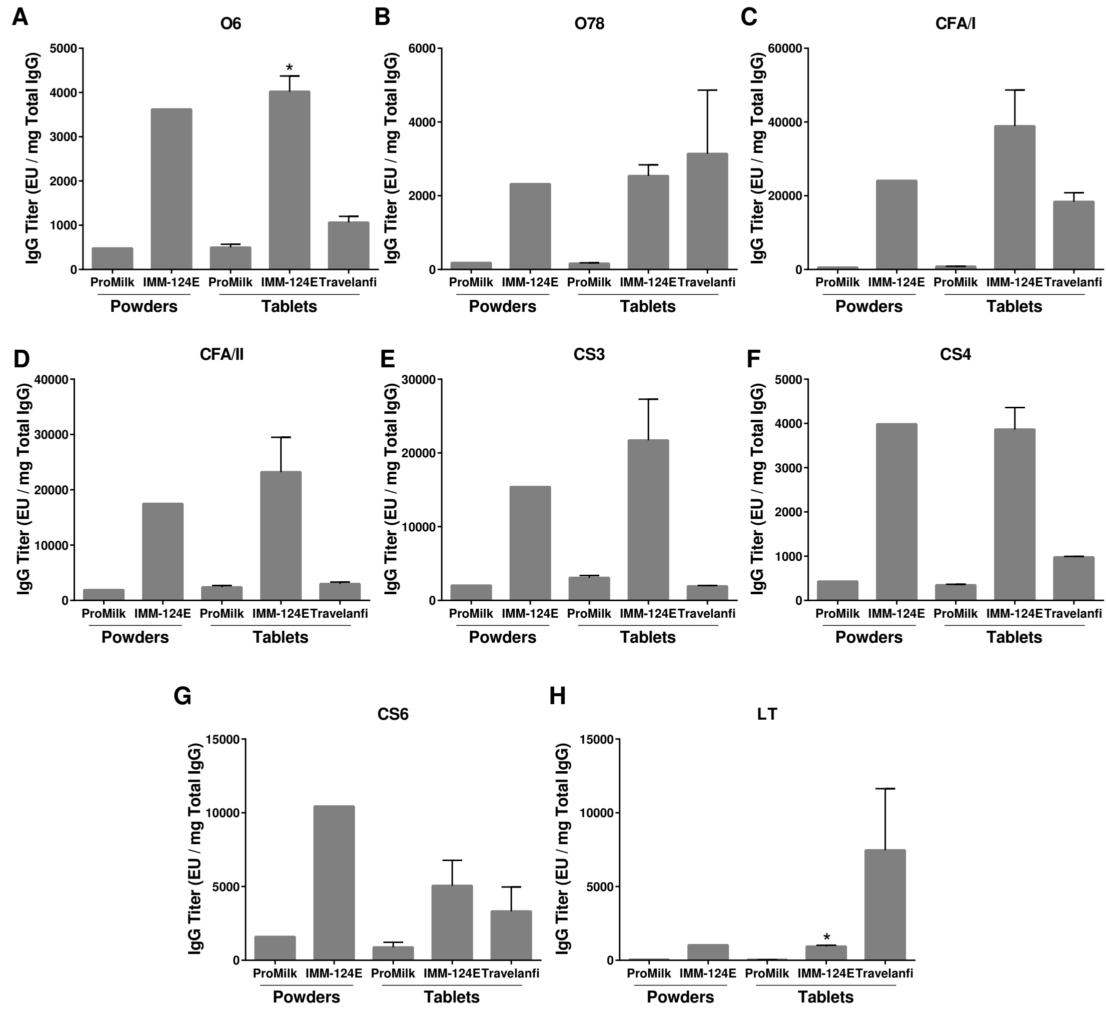

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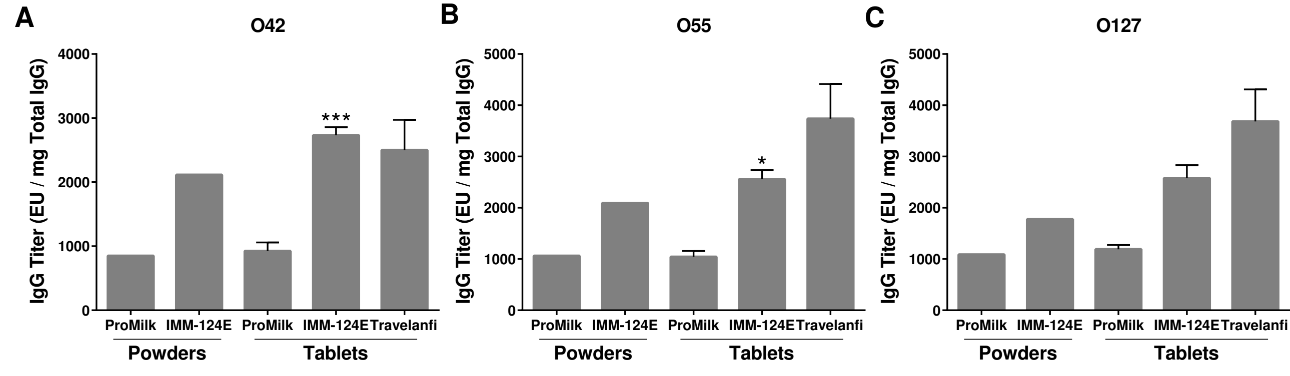

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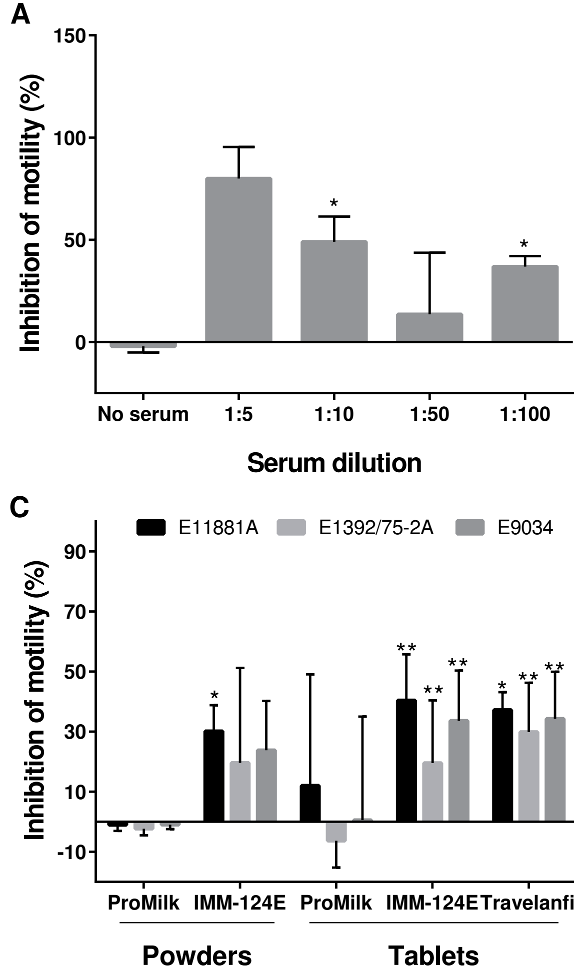

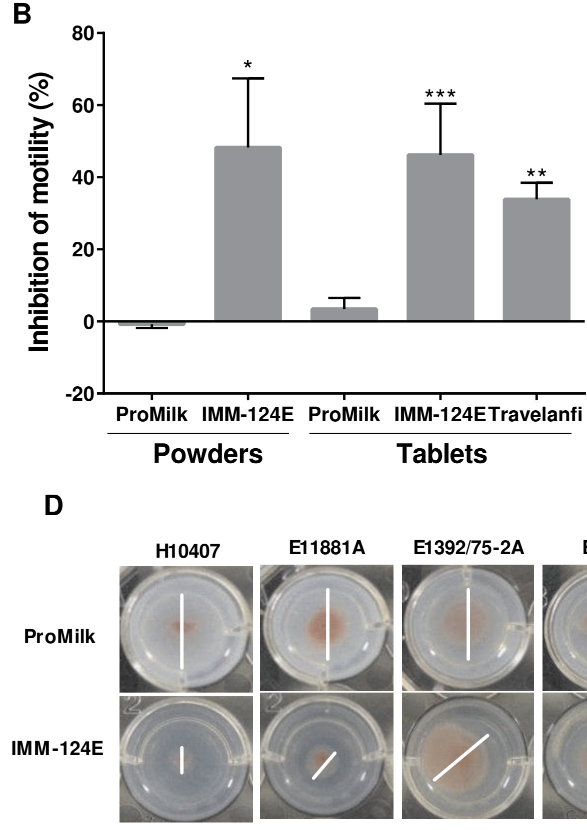

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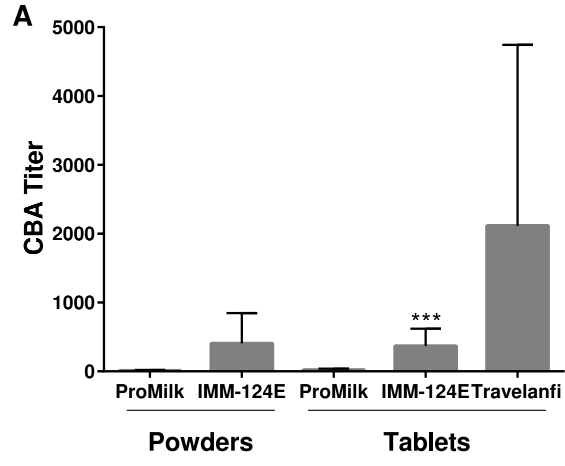

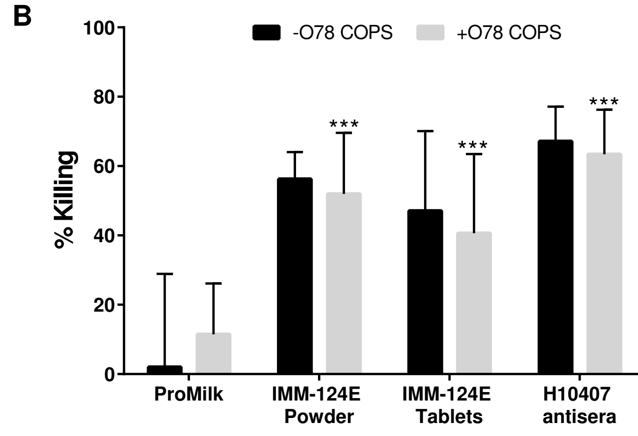

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