Group IIA secreted phospholipase A_2 in human serum kills commensal but not clinical *Enterococcus faecium* isolates

Short title: Normal human serum kills commensal *Enterococcus faecium* isolates.

Fernanda L. Paganelli[1*], Helen L. Leavis[2], Samantha He[1], Nina M. van Sorge[1], Christine Payré[3], Gérard Lambeau[3], Rob J.L. Willems[1], Suzan H.M. Rooijakkers[1].

1. Department of Medical Microbiology, University Medical Center Utrecht, Utrecht University, The Netherlands.
2. Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht University, The Netherlands.
3. Université Côte d'Azur, CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France.

* corresponding author

E-mail: f.paganelli@umcutrecht.nl

Abstract

Human innate immunity employs cellular and humoral mechanisms to facilitate rapid killing of invading bacteria. The direct killing of bacteria by human serum is mainly attributed to the activity of the complement system that forms pores in Gram-negative bacteria. Although Gram-positive bacteria are considered resistant to serum killing, we here uncover that normal human serum effectively kills *Enterococcus faecium.* Comparison of a well-characterized collection of commensal and clinical *E. faecium* isolates revealed that human serum specifically kills commensal *E. faecium* strains isolated from normal gut microbiota, but not clinical isolates. Inhibitor studies show that the human group IIA secreted phospholipase A2 (hGIIA), but not complement, is responsible for killing of commensal *E. faecium* strains in human normal serum. This is remarkable since hGIIA concentrations in 'non-inflamed' serum were considered too low to be bactericidal against Gram-positive bacteria. Mechanistic studies showed that serum hGIIA specifically causes permeabilization of commensal *E. faecium* membranes. Altogether, we find that a normal serum concentration of hGIIA effectively kills commensal *E. faecium* and that hGIIA resistance of clinical *E. faecium* could have contributed to the ability of these strains to become opportunistic pathogens in hospitalized patients.

Importance

Human normal serum contains antimicrobial components that effective kill invading Gram-negative bacteria. Although Gram-positive bacteria are generally considered resistant to serum killing, here we show that normal human effectively kills the Gram-positive *Enterococcus faecium* strains that live as commensals in the gut of humans. In contrast, clinical *E. faecium* strains that are responsible for opportunistic infections

in debilitated patients are resistant against human serum. The key factor in serum responsible for killing is group IIA secreted phospholipase A2 (hGIIA) that effectively destabilizes commensal *E. faecium* membranes. We believe that hGIIA resistance by clinical *E. faecium* could have contributed to the ability of these strains to cause opportunistic infections in hospitalized patients. Altogether, understanding mechanisms of immune defense and bacterial resistance could aid in further development of novel anti-infective strategies against medically important multidrug resistant Gram-positive pathogens.

Introduction

The human immune system is essential to protect us against invading bacterial infections. The first line of immune defense is comprised of cellular and humoral factors that together fight infections in the first minutes to hours of an infection against bacteria. Phagocytic cells, such as neutrophils are able to engulf invading bacteria by bacterial recognition, which is enhanced by bacterial labeling with serum factors as antibodies and complement activation products (1). However, human serum also harbors various antimicrobial proteins and peptides that can directly lyse bacteria without the help of immune cells (2). This serum bactericidal activity is mainly effective against Gram-negative bacteria that are sensitive for the pore-forming Membrane Attack Complex (MAC) of the human complement system (3). For long it has been known that Gram-positive bacteria are resistant to this 'serum bactericidal activity'. It is generally assumed that all Gram-positive bacteria (both pathogenic and non-pathogenic) protect themselves against complement-induced pore formation via a thick layer of peptidoglycan that surrounds the bacterial membrane (4). We here found that normal human serum selectively kills commensal *E. faecium* strains,

76 whereas disease-associated *E. faecium* strains remain viable.

77 *Enterococcus faecium* is a common inhabitant of the gut of mammals, birds and
78 insects (5, 6). While *E. faecium* colonization is harmless in healthy individuals, this
79 bacterium can cause serious infections in immunocompromised patients, such as
80 bacteremia and endocarditis (7). In fact, multidrug resistant *E. faecium*, most notably
81 vancomycin-resistant *E. faecium* (VRE), has emerged as an important cause of
82 nosocomial infections worldwide (8). Recent work showed that clinical *E. faecium*
83 isolates are genetically distinct from commensal strains (9). Whole genome
84 comparison and functional assays identified various virulence factors and
85 carbohydrate metabolism gene clusters that were enriched in clinical isolates and that
86 could contribute to successful niche adaptation in hospitals (9-12). Hospitalized
87 patients suffering from clinical *E. faecium* infections, frequently have a severely
88 compromised cellular immunity. Yet, innate humoral immunity could still aid in *E.*
89 *faecium* infection control (13). To what extent this is relevant in this infection type
90 (by clinical *E. faecium* strains), is currently not completely understood (14-19).

91 Screening of a collection of commensal and multidrug resistant clinical *E. faecium*
92 strains revealed that normal human serum specifically kills commensal strains. We
93 found that the group IIA secreted phospholipase A2 (hGIIA) is key to the bactericidal
94 activity of serum and that resistance to serum hGIIA could have contributed to the
95 development of clinical *E. faecium* isolates.

96

97 ## Results

98 **Human serum kills commensal but not clinical *Enterococcus faecium* strains.**

99 While human serum effectively kills Gram-negative bacteria (20), it is generally
100 accepted that serum does not kill pathogenic and non-pathogenic Gram-positives (21).

101 Serendipitously however, we discovered that a commensal strain of *E. faecium*
102 (E1007, isolated from feces of a healthy individual) was effectively killed by human
103 serum. We incubated 10^5 exponential phase *E. faecium* E1007 with normal human
104 serum (pooled from 20 healthy volunteers) and quantified bacterial survival via
105 colony enumeration on agar plates (Fig 1A). Within 30 minutes, 10% human serum
106 could completely kill 10^5 E1007 bacteria. In contrast, a clinical *E. faecium* strain
107 (E1162, isolated from blood of a hospitalized patient) was fully resistant to serum
108 killing in a similar assay. Based on these results, we extended these analyses to a
109 broader panel of genomically well-characterized clinical and commensal *E. faecium*
110 isolates. We selected clinical *E. faecium* strains isolated from hospitalized patients
111 and compared their serum susceptibility to a selection of commensal *E. faecium*
112 isolated from healthy individuals or animals. Exposure of all 19 strains (described in
113 Table 1) to 25% human serum revealed that human serum specifically kills
114 commensal strains by 70% reduction on average, but not clinical *E. faecium* strains,
115 which survive for 98% (Fig 1B).
116

117 **Complement does not contribute to serum-mediated killing of commensal *E.***
118 ***faecium.***

119 To investigate how human serum kills commensal *E. faecium* strains, we first used
120 several approaches to inactivate the complement system. Bactericidal activity of the
121 complement system is mediated by the Membrane Attack Complex (MAC), a ring-
122 structured pores consisting of proteins C5b, C6, C7, C8 and multiple copies of C9
123 (C5b-9). Formation of pores in bacterial membranes occurs rapidly following an
124 enzymatic chain reaction on the cell surface. Initial experiments seemed to suggest
125 that complement indeed played a role in killing of *E. faecium*. For instance, we found

126 that *E. faecium* killing could be blocked by exposing the serum to 56ºC (heat-
127 inactivated (HI) serum), a method commonly used to inactivate certain heat-labile
128 complement components (22) (Fig 2A-B). Furthermore, addition of the chelating
129 agent (Ethylenediaminetetraacetic acid - EDTA), known to block the complement
130 reaction (23), also interfered with serum killing (Fig 2A, B). However, when we used
131 more specific inhibitors to block the complement reaction, we found that these
132 inhibitors did not affect serum-mediated killing of *E. faecium* (Fig 2A). For instance,
133 application of C3 inhibitor CP40 (24) and C5 inhibitor OmCI (25) did not block
134 bactericidal activity (Fig 2A), although both inhibitors effectively blocked MAC
135 deposition on the bacterial surface (Fig 2B). Resistance of the clinical *E. faecium*
136 strain E1162 was not affected by any of the serum treatments (S1 Fig). From these
137 data, we concluded that killing of commensal *E. faecium* in human serum is not
138 mediated by complement, but by another heat-sensitive and divalent cation-dependent
139 factor (26).
140

141 **hGIIA is essential for serum killing of commensal *E. faecium*.**

142 Next, we decided to test the role of hGIIA (14 kDa), which catalytic function is
143 calcium-dependent (27). hGIIA belongs to the secreted PLA2 family of enzymes
144 present in various genomes, from humans to snakes, invertebrates, plants, fungi and
145 even bacteria that hydrolyze membrane phospholipids at the *sn-2* position (28).
146 Although immune cell derived hGIIA was previously identified as a bactericidal
147 component against several Gram-positive bacteria(29), hGIIA concentrations in
148 normal serum are much lower than the reported bactericidal concentrations for most
149 Gram-positive bacteria (30-33). In our pooled human serum, we observed a
150 concentration of hGIIA of 5 ng/ml (0.3 nM), similar to those described previously

151 (31, 34) (S2 Fig). Nevertheless, we tested whether a specific hGIIA inhibitor
152 (LY311727, Sigma-Aldrich) could block killing of commensal *E. faecium* in human
153 serum. We observed that the inhibitor blocks the killing activity in a dose dependent
154 manner (Fig 3A) and at different concentrations of human serum (Fig 3B). Moreover,
155 the hGIIA inhibitor blocked killing of all tested commensal *E. faecium* strains (Fig
156 3C). Complementary, we could restore killing of commensal *E. faecium* in heat-
157 inactivated serum by reconstituting serum with pure recombinant human hGIIA (Fig
158 3D). In summary, we found that hGIIA is the key player in killing of commensal *E.*
159 *faecium* strains by normal human serum.
160

161 **Serum hGIIA causes membrane destabilization in commensal *E. faecium***

162 Finally, we studied how hGIIA in serum causes bacterial killing. hGIIA acts by
163 hydrolyzing membrane phospholipids in bacterial membranes, whereas host cells are
164 highly resistant to its activity at normal physiological concentrations (30). Here, we
165 used a flow cytometric approach to study whether hGIIA can induce membrane
166 damage to commensal *E. faecium* strains. Bacteria were incubated with human serum
167 in the presence of the DNA dye (sytox green) that only binds DNA and RNA when
168 bacterial membranes are damaged (35). While untreated *E. faecium* cells remain sytox
169 negative, 90% in average of the commensal *E. faecium* population became sytox
170 positive after 30 min incubation with serum concentrations equal or above 10% (Fig
171 4B). No increase in sytox intensity was observed in the clinical strain E1162 (Fig 4A-
172 B). Flow cytometric quantification of sytox influx showed a concentration-dependent
173 effect of serum at inducing membrane damage of commensal, but not clinical *E.*
174 *faecium* strains (Fig 4B). Confocal microscopy confirmed membrane damage of the
175 commensal *E. faecium* E1007 strain but not clinical E1162 strain by human serum

176 (Fig 4C) with different DNA dyes (syto9 and propidium iodide). Furthermore, we
177 found that the serum-induced membrane permeabilization of the E1007 strain was
178 mediated by hGIIA, since EDTA and hGIIA inhibition blocked the observed sytox
179 influx (Fig 4D). Finally, we found that all tested commensal, but not clinical *E.*
180 *faecium* isolates are sensitive to membrane permeabilization via hGIIA in human
181 serum (Fig 4E).
182 Altogether we conclude that hGIIA is the principal component of human serum,
183 which effectively kills commensal *E. faecium* at low concentrations (in the
184 subnanomolar nM range) by destabilization of the bacterial membrane.
185

186 **Discussion**

187 Novel anti-infective strategies are pivotal to curtail the emergence of multidrug-
188 resistant pathogens. Besides development of direct antibacterial compounds, drugs
189 acting at the level of interaction between microbes and the immune system could be
190 promising. Previous studies revealed that *E. faecium* can adopt distinct lifestyles
191 (commensal and pathogenic), which are represented by two distinct genomic clades
192 that differ in genetic polymorphisms and gene repertoire (9, 10). Here we identify that
193 these two clades display distinct susceptibility to killing by normal human serum.
194 Given the genetic variances in genes that are conserved in commensal and clinical
195 strains as well as the differences in gene repertoire between clinical and commensal
196 *E. faecium* strains, it is likely that gene expression and/or differences in gene content
197 (a result of gene gain and loss) explain this difference in hGIIA resistance between
198 commensal and clinical strains. Further investigation is needed to identify the
199 bacterial factors that contribute to *E. faecium* resistance or susceptibility to serum, but
200 genes related to changes in bacterial surface charge and involved in lipid synthesis in

201 bacteria (36, 37) would be good candidates to be investigated, since hGIIA binding is
202 directly influence by the negative charge of the bacterial surface and phospholipids
203 modifications (30). We hypothesize that the development of human serum resistance
204 may have contributed to the decisive step for *E. faecium* to evolve into a relevant
205 pathogen. On the other hand, the ability of hGIIA to kill commensal Gram-positives
206 can be an evolutionary strategy of the innate immune system to contain commensal
207 bacteria in their specific niches and preclude invasion of sterile tissues.
208 Our study also highlights an important role for hGIIA in the humoral immune
209 response against *E. faecium*. While other groups reported that high concentrations of
210 hGIIA (produced locally in lungs and tears (38, 39) or in serum under septic shock
211 conditions) (40, 41) could disrupt certain Gram-positive bacteria, its importance in
212 normal serum or plasma has not been recognized in physiological concentrations,
213 besides against *Listeria monocytogenes* (34). We observed restoration of killing of
214 commensal *E. faecium* when recombinant hGIIA was added in physiological
215 concentrations in heat-inactivated human serum. Although our data indicate that
216 hGIIA is the major factor in serum responsible for the killing phenotype observed
217 against commensal *E. faecium* strains, further studies are necessary to identify the
218 specific conditions in serum that facilitate hGIIA action.
219 hGIIA is known for its ability to hydrolyze bacterial membrane phospholipids, which
220 is a major structural component of the bacterial cell wall (27). Its relevance during
221 infections stems from *in vivo* studies, in which mice overexpressing human hGIIA
222 better control infections by group B Streptococcus, *Staphylococcus aureus* and
223 *Bacillus anthracis* than their control littermates (41-44). Furthermore, the fact that
224 pathogenic Gram-positive bacteria developed resistance against hGIIA is an
225 indication for its relevance *in vivo* (44). Future work is needed to unravel whether

226 serum sensitivity to hGIIA mediated killing is specific for commensal *E. faecium*
227 strains or whether other non-pathogenic Gram-positives may be killed by normal
228 human serum as well. Thus far, non-pathogenic Gram-positives appear resistant to
229 serum killing (21). Only *B. anthracis* was previously shown to be sensitive to normal
230 serum, however this was fully dependent on growth inhibition by transferrin-mediated
231 iron deprivation (45). hGIIA is an acute phase reactant protein, whose serum
232 concentration upon bacterial infection can increase up to hundred fold, which is high
233 enough to kill some Gram-positive pathogens (31). Since hGIIA is able to kill
234 commensal variants of a clinically relevant multi-resistant pathogen, and since hGIIA
235 resistance can be an important mechanism of bacterial escape, strategies based on the
236 use of components of the innate immune system such as hGIIA and even variants of
237 this latter may be developed to fight against multidrug-resistant bacteria. In
238 conclusion, the findings presented here not only provide fundamental knowledge
239 about how the innate immune system kills bacterial cells, it also opens up new
240 therapeutic routes to boost immune clearance of bacteria through conversion of serum
241 resistance.
242

243 ## Materials and Methods

244 **Bacterial strains and growth conditions**

245 The 19 *E. faecium* strains used in this study are from our laboratory collection
246 (department of medical microbiology, UMC Utrecht) and were previously
247 characterized bacteria isolated from healthy and hospitalized humans or animals
248 (Table 1) (9, 46). *E. faecium* was grown at 37ºC for 24 hours in Trypticase soy agar II
249 (TSA) plates supplemented with 5% sheep blood (BD Biosciences) and tryptic soy
250 broth media (TSB; Oxoid) when indicated.

251

252 **Serum, plasma and inhibitors**

253 Normal human serum (HS) was generated at the department of medical microbiology,
254 UMC Utrecht. As previously described (21), whole blood was drawn via venous
255 puncture from 20 healthy volunteers who provided written informed consent in
256 accordance with the Declaration of Helsinki and approval was obtained from the
257 medical ethics committee of the UMC Utrecht. Following collection via venous
258 puncture, blood was clotted for 15 min at room temperature. Blood was centrifuged
259 (10 min at 2700 × g at 4°C) and serum (supernatant) was collected, pooled and frozen
260 in small aliquots before storage at −80°C. Heat-inactivated (HI) serum was prepared
261 by incubating serum at 56ºC for 30 min. When indicated, EDTA (10 mM), C3
262 inhibitor compstatin (CP40) (24) (10 µg/ml), C5 inhibitor OmCI (25) (10 µg/ml),
263 group IIA secreted phospholipase A2 (hGIIA) inhibitor LY311727 (50 µM; Sigma-
264 Aldrich Ldt.) were used. Pure recombinant hGIIA was prepared in *E. coli* as
265 described (44, 47). hGIIA concentration in human serum was quantified using the
266 human sPLA2-IIA enzyme linked immunosorbent assay (ELISA) kit from Cayman
267 Chemicals (Ann Arbor, MI).

268

269 **Serum bactericidal assays**

270 *E. faecium* strains were grown in 4 ml TSB to optical density 660 (OD660) 0.4 from
271 an overnight culture in TSA plates supplemented with 5% sheep blood (BD
272 Biosciences). Bacteria were centrifuged and resuspended in sterile RPMI (Gibco). For
273 each specific condition, 10^5 bacteria were incubated with human serum (at the
274 indicated concentrations, with or without inhibitors) in sterile round-bottom 96-wells
275 plates (Greiner) under shaking conditions for 30 min at 37ºC. Killing was evaluated

276 by serial dilution of samples in RPMI and subsequent plating onto TSA plates
277 supplemented with 5% sheep blood. Following overnight incubation at 37ºC,
278 surviving bacteria were quantified by counting the number of colony forming units
279 (CFU). Killing was measured by comparison of total colony forming units after 30
280 min incubation in the control (RPMI) in relation to 25% human serum or other
281 described condition. Bactericidal assays were performed at least in duplicate per
282 condition.
283
284 **Bacterial membrane permeabilization**
285 Bacterial membrane permeability was analyzed by flow cytometry and confocal
286 microscopy. Following incubation of *E. faecium* with serum, the membrane
287 impermeable nuclei acid dye sytox green (Life technology, 1:5000 (v:v)) was added
288 to the samples. Sytox green staining was quantified by flow cytometry on a BD
289 FACSVerse (Becton Dickinson, San Jose, CA, USA, 488 nm laser). Bacteria were
290 gated based on forward and side scatter properties and fluorescence of 10.000
291 bacterial cells was quantified. Results were analyzed with FlowJo (version v10).
292 Based on the buffer negative control condition (RPMI), a threshold was set to
293 determine the increase of sytox green signal in the population. The increase of sytox
294 signal was represented as the percentage of the total population that was stained with
295 sytox green (see results Fig 4A for example of flow cytometry plots). For confocal
296 microscopy, bacteria were incubated with 25% serum as described above in the
297 presence of syto9 (1.5 µl) and propidium iodide (1.5 µl) (both from BacLight bacteria
298 viability kit, Life Technologies). After 10 min incubation at room temperature,
299 bacteria were mounted in a glass slide with ProLong antifade mountant (Life
300 Technologies), followed by fluorescence analysis in the confocal microscope (Leica

301 SP5). Syto9 and propidium iodide were excited at 488 nm. Pictures were taken at 40x
302 magnification and optical zoom of 3x.
303
304 **Surface deposition of activated complement products**
305 Deposition of C3b molecules or C5b-9 complexes on *E. faecium* surface was
306 quantified by flow cytometry as previously described (21). *E. faecium* was incubated
307 with human serum (or plasma, with or without inhibitors) for 30 min at 37ºC, shaking.
308 After washing, bacteria were incubated with FITC-conjugated anti-C3 antibody
309 (1 µg/ml, Protos Immunoresearch) or mouse anti-C5b-9 antibody (1 µg/ml, aE11
310 Santa Cruz) in PBS-1% BSA for 30 min at 4°C. For C5b-9 detection, a subsequent
311 incubation with FITC-conjugated goat anti-mouse IgG antibody (1 µg/ml, Dako) was
312 performed (30 min at 4°C). Bacteria were washed once more and fluorescence of
313 10.000-gated bacteria was quantified by flow cytometry using a FACSVerse flow
314 cytometer (Becton Dickinson, San Jose, CA, USA) (48).
315

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320

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Tables

Table 1. Ecological origin and clade assignment of 19 *E. faecium* strains.

Strains	Other name	Clade[1]	Country[2]	Year	Source	Isolation site	Reference
E0333	EnGen0013	A1	ISR	1997	Hospitalized patient	Blood	(9)
E0745	E0745	A1	NL	2000	Hospitalized patient	Feces	(49)
E1162	E1162	A1	FR	1997	Hospitalized patient	Blood	(50)
E1321	EnGen0054	A1	IT	1999	Hospitalized patient	Catheter	(9)
E1392	EnGen0016	A1	GBR	2000	Hospitalized patient	nd	(9)
E1644	EnGen0051	A1	GER	2002	Hospitalized patient	nd	(9)
E1731	EnGen0036	A1	TZA	2002	Hospitalized patient	Blood	(9)
E2297	EnGen0034	A1	USA	2001	Hospitalized patient	Urine	(9)
E2560	EnGen0046	A1	NL	2006	Hospitalized patient	Blood	(9)
E0045	EnGen0005	IG	GBR	1992	Health Poultry	Feces	(9)
E0164	EnGen0010	IG	NL	1996	Health Poultry	Feces	(9)
E1573	EnGen0009	A1	BE	1994	Health Bison	Rumen	(9)
E1604	EnGen0028	B	NO	1956	Cheese	-	(9)
E2134	EnGen0043	IG	NL	2004	Health Poultry	nd	(9)
E4215	EnGen0048	IG	SWE	2004	Health Poultry	nd	(9)
E1007	EnGen0015	B	NL	1998	Health Human	Feces	(9)
E1050	EnGen0017	A1	NL	1998	Health Human	Feces	(9)
E1590	EnGen0003	B	IRL	2001	Health Human	Feces	(9)
E3548	EnGen0047	B	NL	2004	Health Human	Blood	(9)

[1]Clade structure was based on the classification described by Paganelli *et al.* (46)

[2]Contries: NL: Netherlands; ISR: Israel; FR: France; IT: Italy; GBR: Great Britain; GER: Germany; TZA: Tanzania; USA: United States; BE: Belgium; NOR: Norway;

463 SWE: Sweden; IRL: Ireland.

464

465 ## Figure Captions

466 **Figure 1. Human serum kills commensal, but not clinical *E. faecium* isolates.**

467 (A) Comparison of bacterial survival at different concentrations of pooled normal
468 human serum of clinical *E. faecium* strain E1162 (clinical) and commensal *E. faecium*
469 strain E1007 (commensal). (B) Killing of *E. faecium* isolates originating from
470 hospitalized patients (clinical), healthy animals (commensal animal) and healthy
471 humans (commensal humans) in 25% pooled human serum. Killing was quantified by
472 comparison of total colony forming units after 30 min incubation in the buffer
473 negative control (RPMI) compared to 25% human serum. Epidemiological details of
474 *E. faecium* strains are listed in table 1.

475

476 **Figure 2. Complement does not contribute to serum-mediated killing of**
477 **commensal *E. faecium.***

478 (A) Killing of commensal *E. faecium* strain E1007 was measured by bacterial
479 viability on blood agar plates after 30 min incubation at the indicated conditions. (B)
480 Complement system activity in human serum was measured by C5b-9 deposition on
481 bacterial surface of clinical isolate E1162.

482

483 **Figure 3. hGIIA is essential for serum killing of commensal *E. faecium.***

484 (A) Killing of commensal *E. faecium* strain E1007 in 25% pooled human serum is
485 inhibited by hGIIA inhibitor (LY311727) in a dose-dependent manner. (B) Killing of
486 commensal *E. faecium* strain E1007 in different concentrations of human serum
487 incubated with or without hGIIA inhibitor (LY311727). (C) Killing of *E. faecium*

488 commensal strains E1050, 1590, E3548 and E1007 in 25% pooled human serum with
489 or without 50 μM of hGIIA inhibitor (LY311727), respectively. (D) Killing of *E.*
490 *faecium* commensal strains E1050, 1590, E3548 and E1007 in 25% pooled heat-
491 inactivated (HI) human serum without or with recombinant hGIIA, respectively.
492 Killing was quantified by bacterial viability on blood agar plates after 30 min
493 incubation in the different conditions.
494
495 **Figure 4. Serum hGIIA causes membrane permeabilization in commensal *E.***
496 ***faecium*.**
497 (A) Representative flow cytometry plots of sytox green influx (switch to the right) of
498 commensal *E. faecium* isolate E1007 incubated with RPMI (buffer) and clinical
499 (E1162) and commensal (E1007) strains incubated with 25% pooled human serum.
500 (B) Sytox green influx in E1007 and E1162 using different concentration of human
501 serum. (C) Confocal pictures of clinical (E1162) and commensal (E1007) *E. faecium*
502 strains incubated with RPMI (buffer) or 25% human serum in the presence of syto9
503 (in green) representing live cells and propidium iodide (in red) representing damaged
504 bacterial membrane. *E. faecium* membrane permeabilization of clinical strain E1162
505 and commensal strain E1007 was measured by sytox green influx in the described
506 conditions. (D) Sytox green influx upon incubation in serum in the absence or
507 presence of hGIIA inhibitors and upon restoration in heat-inactivated (HI) human
508 serum with recombinant hGIIA. (E) Sytox green influx in *E. faecium* clinical strains
509 E2560, E0745, E1162 and commensal strains E1050, E1590, E3548, E1007 in RPMI
510 (buffer), 25% pooled human serum without or with 50 μM of hGIIA inhibitor
511 (LY311727) or heat-inactivated (HI) human serum with 5 ng/ml recombinant hGIIA,
512 respectively.

513

514 ## Supplementary Material Captions

515 **S1 Figure. Complement system activity in human serum.** A. Killing of clinical *E.*
516 *faecium* strain E1162 was measured by cell viability on blood agar plates after 30 min
517 incubation on the indicated conditions. B. Inhibition of complement system was
518 measured by C3b deposition on bacterial surface.

519

520 **S2 Figure. hGIIA concentration in pooled normal human serum.** hGIIA
521 concentration in pooled normal human serum used in all assay was measured by Elisa
522 kit (Cayman).

523

Figure 1.

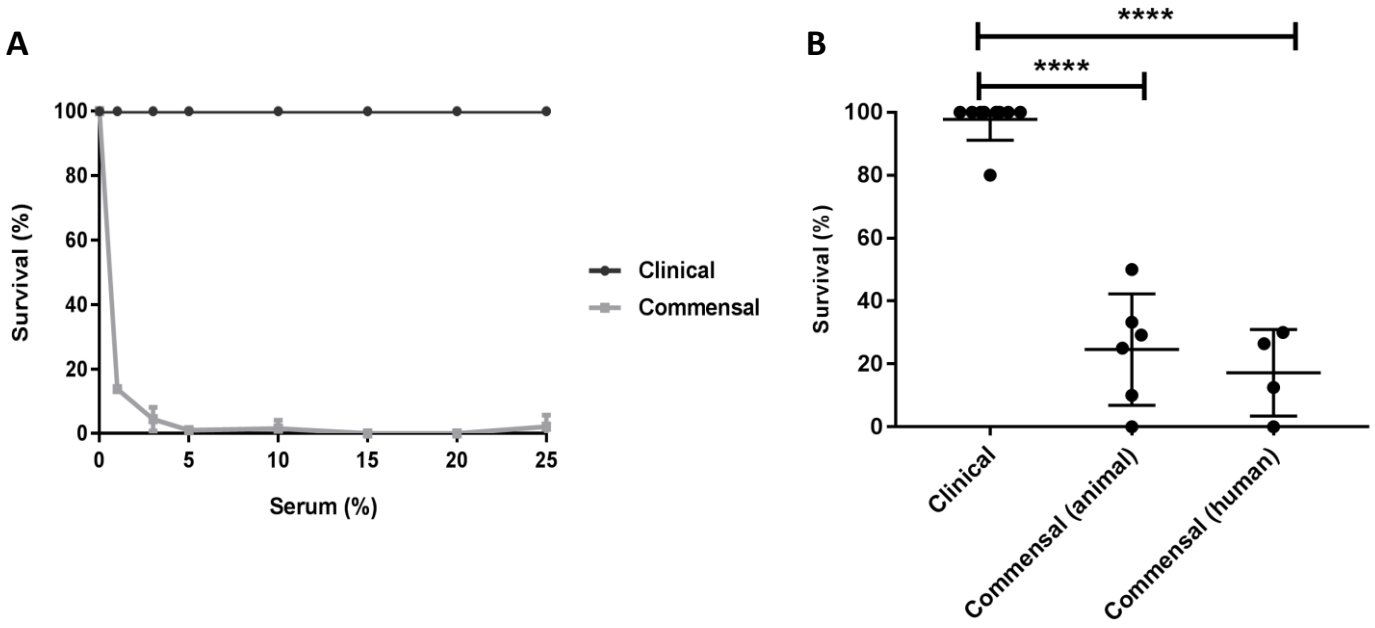

A
Survival (%)
100
80
60
40
20
0
0
5
10
15
20
25
Serum (%)
Clinical
Commensal
B
****
****
Survival (%)
100
80
60
40
20
0
Clinical
Commensal (animal)
Commensal (human)

Figure 2.

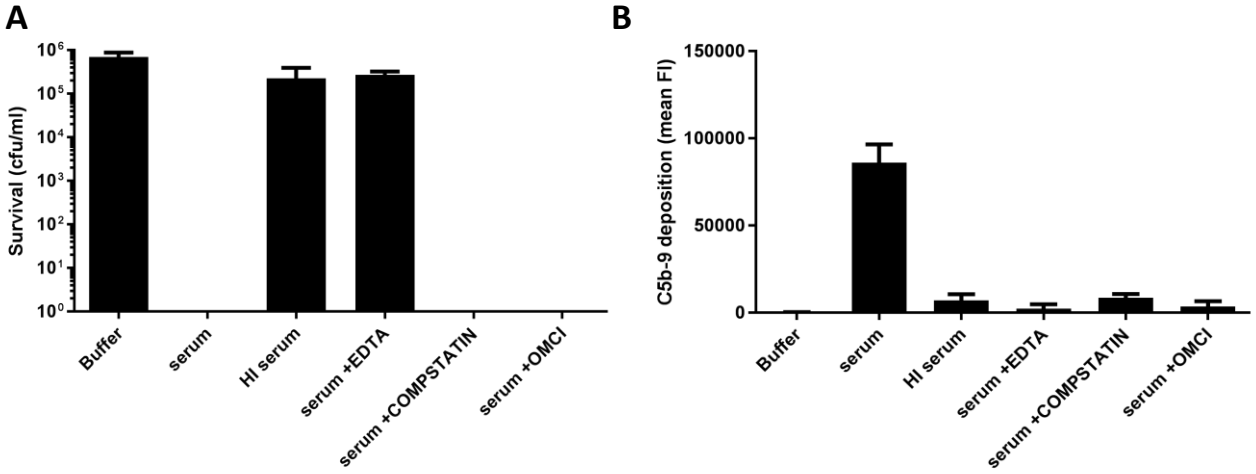

A
Survival (cfu/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Buffer
serum
HI serum
serum +EDTA
serum +COMPSTATIN
serum +OMCI
B
C5b-9 deposition (mean FI)
150000
100000
50000
0
Buffer
serum
HI serum
serum +EDTA
serum +COMPSTATIN
serum +OMCI

Figure 3.

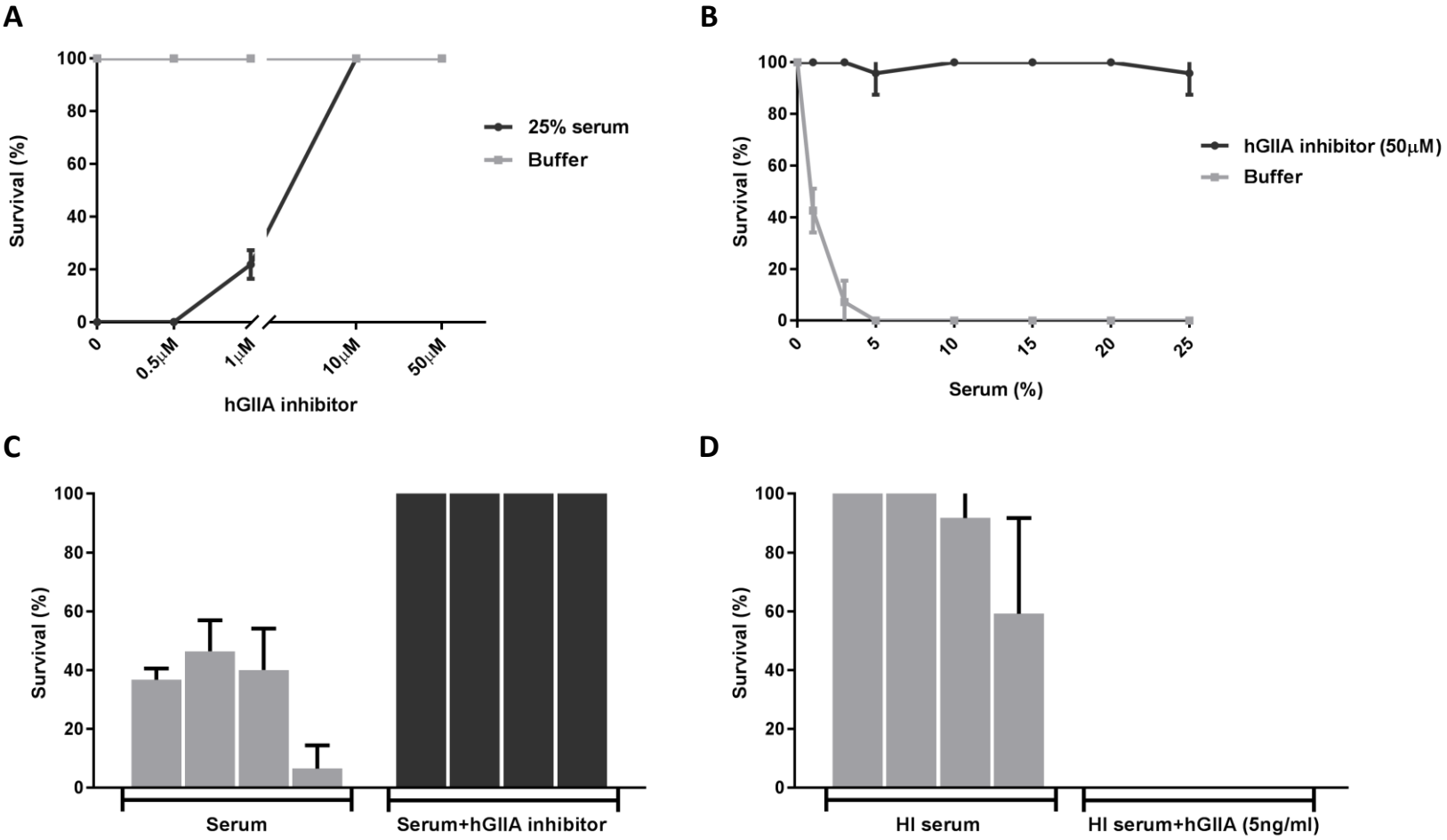
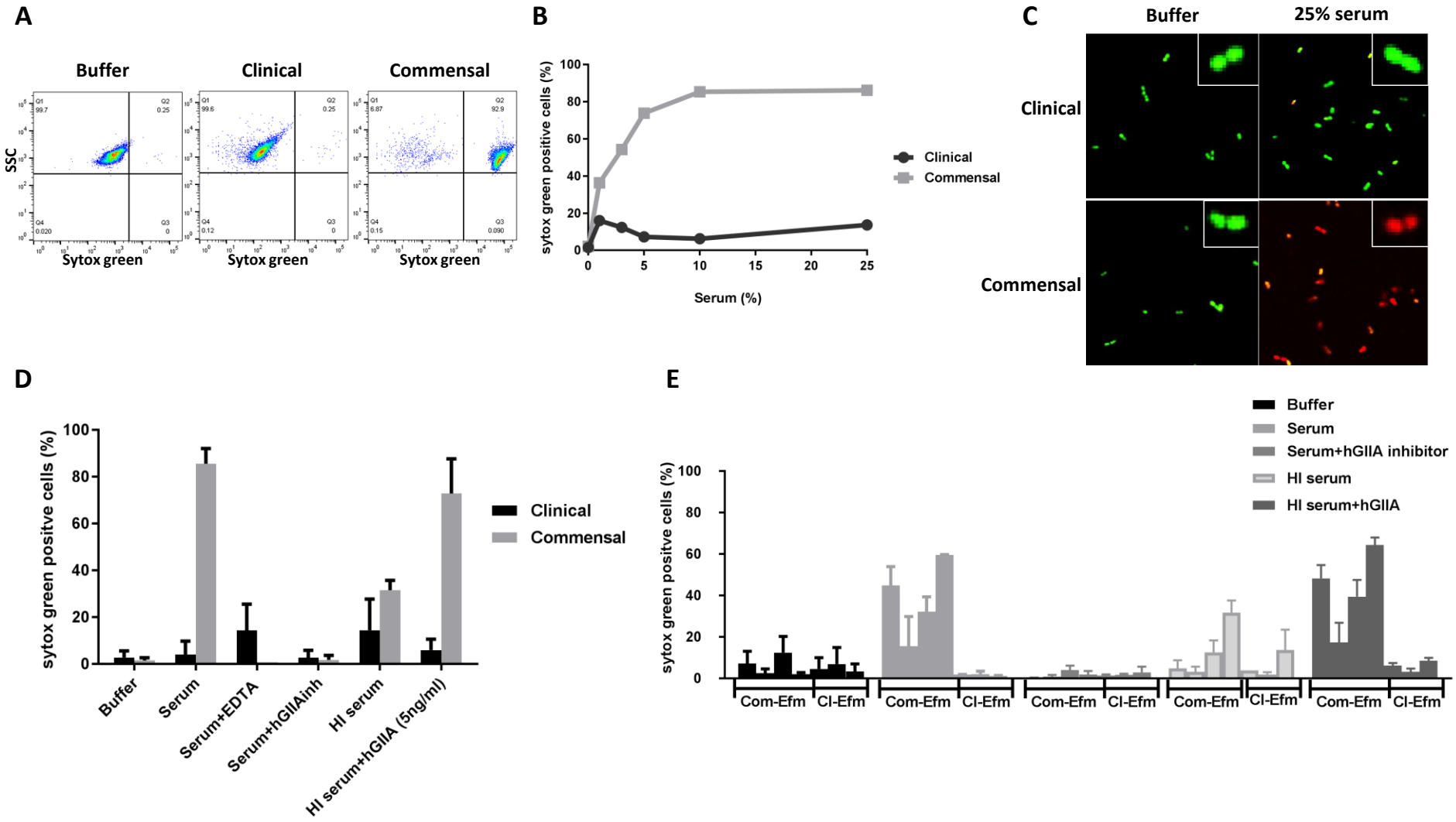

A
Survival (%)
100
80
60
40
20
0
0
0.5μM
1μM
10μM
50μM
hGIIA inhibitor
25% serum
Buffer
B
Survival (%)
100
80
60
40
20
0
0
5
10
15
20
25
Serum (%)
hGIIA inhibitor (50μM)
Buffer
C
Survival (%)
100
80
60
40
20
0
Serum
Serum+hGIIA inhibitor
D
Survival (%)
100
80
60
40
20
0
HI serum
HI serum+hGIIA (5ng/ml)

Figure 4.


A
Buffer
Clinical
Commensal
SSC
Sytox green
B
sytox green positive cells (%)
Serum (%)
Clinical
Commensal
C
Buffer
25% serum
Clinical
Commensal
D
sytox green positve cells (%)
Clinical
Commensal
Buffer
Serum
Serum+EDTA
Serum+hGIIAinh
HI serum
HI serum+hGIIA (5ng/ml)
E
sytox green positve cells (%)
Buffer
Serum
Serum+hGIIA inhibitor
HI serum
HI serum+hGIIA
Com-Efm
Cl-Efm