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1 Co-association between Group B *Streptococcus* and *Candida albicans* promotes
2 interactions with vaginal epithelium
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14 Running Head: GBS-*Candida* co-association promotes host interactions
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22 **ABSTRACT**

23 Group B *Streptococcus* (GBS) is a leading cause of neonatal sepsis, pneumonia and
24 meningitis worldwide. In the majority of cases, GBS is transmitted vertically from mother
25 to neonate, making maternal vaginal colonisation a key risk factor for neonatal disease.
26 The fungus *Candida albicans* is an opportunistic pathogen of the female genitourinary
27 tract, and the causative agent of vaginal thrush. Carriage of *C. albicans* has been
28 shown to be an independent risk factor for vaginal colonisation by GBS. However, the
29 nature of interactions between these two microbes is poorly understood. This study
30 provides evidence of a reciprocal, synergistic interplay between GBS and *C. albicans*
31 that may serve to promote their co-colonisation of the vaginal mucosa. GBS strains
32 NEM316 (serotype III) and 515 (Ia) are shown to physically interact with *C. albicans*,
33 with bacteria exhibiting tropism for candidal hyphal filaments. This interaction enhances
34 association levels of both microbes with vaginal epithelial cell line VK2/E6E7. The ability
35 of GBS to co-associate with *C. albicans* is dependent upon expression of hyphal-
36 specific adhesin Als3. In turn, expression of GBS antigen I/II family adhesins (Bsp
37 polypeptides) facilitates this co-association and confers upon surrogate *Lactococcus*
38 *lactis* the capacity to exhibit enhanced interactions with *C. albicans* on vaginal
39 epithelium. As genitourinary tract colonisation is an essential first step in the
40 pathogenesis of GBS and *C. albicans*, the co-association mechanism reported here
41 may have important implications for risk of disease involving both of these pathogens.

42 **INTRODUCTION**

43 *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) is a leading cause of
44 invasive disease (sepsis, pneumonia, meningitis) in neonates, and is responsible for life
45 threatening infections in elderly and immune-compromised individuals (1-3). GBS is an
46 opportunistic pathogen of the female genitourinary (GU) tract, with a carriage rate in
47 Western countries of approximately 30% (2). The primary route of transmission to
48 neonates is from the mother during or preceding birth, with transmission rates estimated
49 at up to 50% (2). Of those neonates that are colonised, about 1% develop severe GBS
50 disease, resulting in significant infant morbidity or mortality (2, 4).

51 A variety of proteins have been identified on the surface of GBS that may promote
52 colonisation of host mucosae. These include pili (5), alpha C protein (6), BibA (7),
53 serine-rich repeat proteins (Srr1/2) (5, 8), FbsA (9), Lmb (10), and the recently
54 characterised antigen I/II (AgI/II) family proteins, designated BspA-D (11, 12). Many of
55 these surface proteins have been shown to target receptors expressed directly on the
56 cervical or vaginal epithelia, while others bind extracellular matrix (ECM) proteins such
57 as collagen, fibrinogen, fibronectin or laminin (5, 8-10). An additional colonisation
58 strategy for GBS, but one that remains poorly understood, is via interactions with other
59 members of the vaginal microbiota. It is widely accepted that a 'healthy' vaginal
60 microbiota is dominated (ca. 70%) by the genus *Lactobacillus*, but Gram-positive
61 bacteria (e.g. streptococci, staphylococci), Gram-negative bacteria (e.g. *Escherichia*
62 *coli*) and yeasts (e.g. *Candida albicans*) are also frequently isolated (13). Of particular
63 relevance to GBS colonisation is a growing body of evidence indicating an association
64 with fungus *C. albicans*. In both developed and developing countries, vaginal carriage of

65 *C. albicans* has been shown to be an independent risk factor for vaginal colonisation by
66 GBS (14-18).
67 *C. albicans* accounts for the fourth highest rate of systemic nosocomial infection
68 in the US (19), and as an opportunistic pathogen of the oropharynx and female GU tract
69 is the predominant cause of both oral and vaginal thrush. Key risk factors for *C. albicans*
70 infection are immunosuppression, use of oral contraceptives, hormone therapy,
71 antibiotics, diabetes, and pregnancy (20). A number of colonisation determinants have
72 been implicated in promoting candidal adhesion to and invasion of mucosae. These
73 include proteins that are expressed on the surface of both morphological forms
74 (blastospore and hypha) of *C. albicans* such as Als1, Eap1, Eno1, Pra1 and Tdh1 (21-
75 25). Other major candidal adhesins, including Hwp1, Als3 and Ssa1, are exclusively
76 expressed on the filamentous hyphae (22, 26, 27). Similarly to GBS, epithelial receptor
77 molecules (e.g. CEACAMs, cadherins) or ECM proteins (e.g. fibronectin, laminin) have
78 been identified as targets for these *C. albicans* adhesins (25, 27-29).
79 Synergistic polymicrobial interactions have already been described for *C. albicans*
80 and a number of Gram-positive bacteria. For example, oral bacterium *Streptococcus*
81 *gordonii* produces nutrient by-products that are stimulatory to *C. albicans,* enhancing
82 the length of hyphal filaments (30). In turn, *S. gordonii* benefits from the reduced oxygen
83 environment generated by *C. albicans* metabolism (31). Physical coadhesion between
84 these two microbes also serves to promote retention of *C. albicans* within the oral
85 cavity, the molecular basis of which has been identified as recognition of *C. albicans*
86 adhesin Als3 by *S. gordonii* AgI/II family protein SspB (32). Similar interactions have
87 been reported for *Streptococcus mutans* and *C. albicans*, for which *S. mutans*

88 glucosyltransferase GtfB has been shown to bind mannans on the candidal cell surface,
89 promoting robust cross-kingdom biofilm formation within the oral cavity of rats (33). In
90 addition to niche colonisation, interkingdom interactions may modulate disease
91 progression. *Streptococcus oralis* and *C. albicans* synergise within the oropharynx to
92 promote breakdown of epithelial tight junctions, resulting in enhanced systemic
93 dissemination of *C. albicans* (34, 35). Likewise *Staphylococcus aureus* has a high
94 affinity for binding *C. albicans* hyphae and can 'piggy back' on these filamentous forms
95 as they infiltrate host cells to gain access to deeper tissues (36). Again, staphylococcal
96 recognition of *C. albicans* hyphal protein Als3 is critical for this coadhesion (37).
97 We have recently characterised the AgI/II family polypeptide of GBS designated
98 BspA. Alongside binding to salivary pellicle and vaginal epithelium, BspA was shown to
99 promote coaggregation of GBS strain NEM316 with *C. albicans* under planktonic
100 conditions (12). This study therefore aimed to build on these initial observations and
101 determine in more detail the interkingdom interactions between GBS and *C. albicans*,
102 and to investigate their potential to modulate the colonisation or pathogenic capabilities
103 of these two microbes within the GU tract.
104
105 **RESULTS**
106 **Planktonic interactions of GBS and *C. albicans*.** The first step in exploring the
107 interactions of GBS with *C. albicans* was to confirm their capacity to coaggregate under
108 planktonic conditions. Two strains of GBS were tested that represent two of the most
109 common capsular serotypes associated with neonatal disease: GBS strain 515,
110 capsular serotype Ia, and strain NEM316, capsular serotype III (Table 1). *C. albicans*

111 was fluorescently-labelled with Calcofluor White, while GBS strains were labelled with
112 FITC. Suspensions were then incubated together for 1 h before visualisation by
113 fluorescence microscopy. Both GBS strains were able to coaggregate with *C. albicans*,
114 indicating that these interactions are not restricted to a single capsular serotype (Fig. 1).
115 Furthermore, as reported by (12), GBS strain NEM316 exhibited a tropism for *C.*
116 *albicans* hyphae rather than blastospores. This binding pattern was also apparent with
117 GBS strain 515, although higher levels of association were seen overall with strain
118 NEM316 (Fig. 1). Taken together, these data confirmed that GBS can undergo
119 planktonic interactions with *C. albicans*, but implied that levels of coaggregation may be
120 strain-dependent.
121 **GBS-*C. albicans* interactions with vaginal epithelial cells.** Since GBS and *C.*
122 *albicans* are able to coaggregate, we hypothesised that such interactions could
123 influence the capacity of these microbes to associate with vaginal epithelium. To this
124 end, an *in vitro* assay was developed using vaginal epithelial cell (VEC) line VK2/E6E7.
125 In the first instance, epithelial cell monolayers were exposed either to GBS alone for 1
126 h, or to *C. albicans* for 1 h to initiate hyphae formation, followed by GBS for a further 1
127 h. Numbers of associated GBS were then enumerated by viable count (CFU) from
128 epithelial cell lysates. While GBS strain NEM316 showed higher levels of association
129 (1.51×10^5 CFU/monolayer) compared to strain 515 (7.57×10^4 CFU/monolayer) (Fig. 2),
130 both strains exhibited a strong affinity for the VEC monolayers. However, significantly
131 higher numbers of bacteria were recovered for both strains in the presence of *C.*
132 *albicans*. Numbers of GBS recovered from the epithelium were 1.9-fold higher for strain
133 NEM316 and 2.1-fold higher for strain 515 compared to their respective monospecies

134 samples (Fig. 2). These augmentory effects were verified by confocal microscopy,
135 although a slightly longer incubation period was needed (5 h) to obtain bacterial cell
136 numbers that were of sufficient abundance to be clearly visible (Fig. 3). For
137 monospecies samples, both GBS strains were evenly distributed across the VECs, but
138 numbers of GBS cells were higher per field of view for strain NEM316 than 515 (Fig. 3,
139 columns 1-2). In the presence of *C. albicans*, an increase in the numbers of GBS
140 associated with the VECs was apparent for both strains (Fig. 3, columns 3-4) compared
141 to the monospecies equivalent. This was verified by quantification of GBS biovolume
142 (Fig. 4A). In the presence of *C. albicans*, GBS biovolume levels were 4.6-fold and 2.8-
143 fold higher for strains NEM316 and 515 respectively than their monospecies equivalent
144 (Fig. 4A). Many GBS cells could be seen interacting with *C. albicans* hyphae, which
145 formed extensive mats that overlaid the epithelial monolayers (see white arrows, Fig. 3).
146 However, there was also a visible increase in the numbers of GBS interacting with the
147 epithelium in areas that were not seemingly colonised by *C. albicans* (see red arrows,
148 Fig. 3). This pattern was seen for both GBS strains. Augmentation by *C. albicans* of
149 GBS association with VECs after 5 h was further supported by enumeration of GBS
150 from recovered epithelial lysates, and more strikingly demonstrated the effects with
151 GBS strain 515 (Fig. 4B).
152 To investigate the potential for a reciprocal relationship between GBS and *C.*
153 *albicans*, the effects of GBS on *C. albicans* association with vaginal epithelium were
154 then explored using the same *in vitro* assay. For both strains tested, the presence of
155 GBS resulted in a 4-fold elevation in the levels of *C. albicans* recovered from the VEC
156 monolayers compared to *C. albicans* alone (Fig. 5). These data imply that a synergistic

157 relationship exists between *C. albicans* and GBS, and that each microbe can enhance
158 association of the other with vaginal epithelium.

159 **Role of diffusible signals in GBS-*C. albicans* interactions.** One potential
160 mechanism for the enhanced recovery of both GBS and *C. albicans* when co-cultured
161 with vaginal epithelium might be that each microbe releases some form of diffusible,
162 chemical signal that either stimulates growth of the other or promotes its capacity to
163 associate with VECs. To explore the first possibility, growth of GBS and *C. albicans* in
164 single- or dual-species suspensions were compared. These studies were performed
165 under similar conditions to the *in vitro* cell culture assay, using keratinocyte serum-free
166 medium (K-SFM) and incubation periods of 1-2 h. No significant differences in CFU
167 were seen for either species (Fig. 6A,B), regardless of whether grown in mono- or dual-
168 species conditions. This implied that the presence of *C. albicans* does not affect the
169 overall growth rate of GBS, and vice versa.

170 To determine if diffusible signals were modulating microbial interactions with the
171 vaginal epithelium, GBS was incubated with VEC monolayers in K-SFM, or in spent
172 media harvested from *C. albicans* grown in K-SFM for 1 h in the presence or absence of
173 VECs. After 1 h incubation with VEC monolayers, numbers of associated GBS were
174 enumerated by viable count from epithelial cell lysates. No significant differences were
175 observed between numbers (CFU/monolayer) of GBS recovered across the different
176 conditions (Fig. 7A).

177 For the reciprocal study, *C. albicans* was incubated on VEC monolayers for 1 h,
178 and then suspensions of GBS or K-SFM alone were placed in transwell inserts above
179 the VECs. Viable counts of *C. albicans* were determined after a further 1 h incubation.

180 Again, no significant differences were seen in *C. albicans* association levels with VECs
181 in the presence or absence of GBS (Fig. 7B).
182 One final possibility explored was that GBS or *C. albicans* modulated the
183 permissiveness of VECs to association with the other microbe via an active but contact-
184 dependent mechanism. This was investigated by repeating the association assays
185 using paraformaldehyde-fixed VECs. Fixation reduced the numbers of GBS recovered
186 from the cell lysates overall. Nonetheless, the presence of *C. albicans* again resulted in
187 elevated association levels of GBS (Fig. 7C) and the reciprocal effect was seen for
188 levels of *C. albicans* recovered in the presence of GBS (Fig. 7D). Taken together, these
189 data imply that neither intermicrobial diffusible signals nor active modulation of VEC
190 receptor profile are required for enhanced co-association of GBS or *C. albicans* with
191 vaginal epithelium.
192 **Role of Bsp protein in GBS-*C. albicans* interactions.** Oral streptococci have
193 been shown to promote the colonisation and retention of *C. albicans* within the oral
194 cavity and this is mediated, in large part, by coadhesion between the microbes (31).
195 Having demonstrated similar coadhesion between GBS and *C. albicans*, the next step
196 was to determine the molecular basis for this physical interaction and its contribution to
197 the synergistic effects seen with vaginal epithelium.
198 We have recently shown that AgI/II family protein BspA of GBS strain NEM316
199 promotes coaggregation with *C. albicans* under planktonic conditions (12). We therefore
200 wanted to build on this observation and determine if the Bsp adhesin family was
201 important in GBS augmenting interactions of *C. albicans* with VECs. In the first instance
202 a Δ*bspC* knockout mutant was generated in GBS strain 515, which carries only a single

203 copy of the *bspC* gene (a homologue of *bspA*). This strain displayed only a modest (ca.
204 15%) reduction in association with VECs when compared to parent strain 515 (Fig. 8A).
205 However, it has been reported previously that streptococci can compensate for loss of
206 antigen I/II family proteins by upregulation of alternative adhesins (38). To further
207 explore the role of Bsp adhesins, inhibition studies were therefore performed using
208 specific antisera. Anti-Bsp sera reduced association of wild-type GBS strains NEM316
209 and 515 to VECs by 46% and 63% respectively compared to preimmune control (Fig.
210 8B). Together these data support previous evidence that Bsp adhesins have capacity to
211 promote GBS interactions with vaginal epithelium (12), but indicate that there are
212 additional determinants utilised by GBS for this purpose.
213 In the presence of *C. albicans*, a more significant difference was seen between
214 parent 515 and Δ*bspC* knockout strains. *C. albicans* significantly promoted recovery of
215 both GBS strains from the epithelium compared to their respective monospecies
216 samples. However, numbers of bacteria recovered were approximately 30% lower for
217 mutant strain 515 Δ*bspC* than parent 515 (Fig. 8A). These data imply that BspC plays a
218 role in mediating GBS co-association with *C. albicans*. However, additional adhesins
219 must be involved and may compensate for the lack of BspC in strain 515 Δ*bspC*.
220 Given this apparent adhesin redundancy, surrogate *Lactococcus lactis* strains
221 expressing BspA or BspC were then employed in co-association assays. This allowed
222 the functional properties associated with the individual AgI/II family proteins to be
223 explored in greater detail. For monospecies *L. lactis*, once again only a modest increase
224 was seen in numbers of bacteria recovered from VECs expressing BspA or BspC
225 compared to the empty vector control strain (Fig. 9). However, for dual species

226 samples, recoveries of *L. lactis* strains expressing BspA or BspC were promoted by 1.8-
227 fold or 3-fold respectively by *C. albicans* (Fig. 9), while vector-only *L. lactis* controls
228 were increased only slightly (<0.5-fold). Overall this implies that GBS AgI/II family
229 proteins have capacity to promote GBS association with vaginal epithelium directly, but
230 they likely play a greater role by promoting association via *C. albicans*.

231 **Role of Als3 protein in GBS-*C. albicans* interactions.** A possible receptor for
232 the Bsp proteins of GBS was candidal glycoprotein Als3, since this adhesin is hypha-
233 specific (22) and has been shown to bind the AgI/II family protein SspB of *S. gordonii* to
234 mediate interkingdom interactions (32). A *C. albicans* strain with both alleles of the *als3*
235 gene deleted (39), as well as a corresponding complemented strain (Δ*als3*+*als3*), were
236 used to determine if Als3 is involved in interactions between GBS and *C. albicans*. This
237 was first investigated under planktonic conditions, and levels of coaggregation were
238 determined semi-quantitatively according to numbers of GBS associated with individual
239 hyphae. Both GBS strains exhibited strong interactions with *C. albicans* wild type
240 SC5314 and *C. albicans* Δ*als3*+*als3* strains, with the majority of hyphae recorded as
241 binding 6-20 bacteria or >20 bacteria (Fig. 10). By contrast, the majority of *C. albicans*
242 Δ*als3* hyphae were either devoid of bacterial cells or bound only 1-5 GBS cells (Fig. 10).
243 Thus the expression of Als3 on candidal hyphae is required to mediate strong physical
244 interactions with GBS under planktonic conditions.

245 These various *C. albicans* strains were then used to determine if Als3-mediated
246 interactions were required to modulate GBS association with VECs. Interestingly,
247 numbers of *C. albicans* Δ*als3* associated with VECs were not significantly different from
248 those recovered for wild type SC5314 or *C. albicans* Δ*als3*+*als3* strains. This was

249 contrary to observations made by others (39) in studies with oral epithelium and implies
250 that Als3 may exhibit tissue-specific tropism. Unlike the phenomenon observed with *C.*
251 *albicans* wild type, there was no enhanced association of GBS with *C. albicans* Δ*als3* in
252 the presence of VECs, and numbers of GBS recovered were comparable to those from
253 monospecies samples (Fig. 11). By contrast, complementation of Δ*als3* in strain *C.*
254 *albicans* Δ*als3*+*als3* restored the capacity for *C. albicans* to significantly promote GBS
255 association with vaginal epithelium relative to GBS monospecies samples (Fig. 11).
256 A similar scenario was seen for the reciprocal studies to determine the role of Als3
257 in GBS modulation of *C. albicans* interactions with VECs. Numbers of *C. albicans* Δ*als3*
258 cells recovered from epithelial monolayers were comparable for monospecies samples
259 and dual-species samples incorporating either of the two GBS strains (Fig. 12). By
260 contrast, both of the GBS strains enhanced the recovery of *C. albicans* Δ*als3*+*als3* by
261 2.5-fold (Fig. 12) relative to the monospecies control. These latter effects were similar to
262 those seen previously with *C. albicans* wild type SC5314 (Fig. 5). Thus, Als3 expression
263 by *C. albicans* is required for both GBS and *C. albicans* to modulate co-association with
264 vaginal epithelium.
265 Finally, studies were performed to investigate if Bsp polypeptides of GBS can bind
266 directly to Als3 of *C. albicans*. Again, to avoid potential issues with adhesin redundancy,
267 surrogate expression strains were utilised. A strain of *Saccharomyces cerevisiae* had
268 previously been generated that expresses the small allele of *C. albicans* Als3 on its cell
269 surface (40). This *S. cerevisiae* (Als3+) strain was fluorescently-labelled with FITC,
270 while *L. lactis* strains expressing BspA, BspC or empty vector control were labelled with
271 TRITC. Suspensions were then incubated together for 1 h before visualisation by

272 fluorescence microscopy. No interactions were seen between *S. cerevisiae* (Als3+) and
273 *L. lactis* control (Fig. 13A). By contrast, coggregation could clearly be seen with *S.*
274 *cerevisiae* (Als3+) and *L. lactis* strains expressing either BspA (Fig. 13B) or BspC (Fig.
275 13C). Thus GBS polypeptides BspA and BspC are direct binding partners for Als3 of *C.*
276 *albicans*.
277

278 DISCUSSION

279 Intermicrobial interactions occur at most sites of colonisation within the human
280 body and, according to the National Institutes of Health, biofilms underpin approximately
281 80% of infections (41). In some instances, these interactions have antagonistic
282 outcomes, such as those between *C. albicans* and *Pseudomonas aeruginosa*. Other
283 partnerships are seemingly synergistic in nature, such as the interactions between *C.*
284 *albicans* and *S. gordonii*, *S. oralis*, *S. mutans* or *Staph. aureus* (32, 35, 37, 42). Several
285 studies have reported co-occurrence of GBS and *C. albicans* within the GU tract (14-
286 18), and we recently provided evidence for coaggregation between these two microbes
287 (12). The aims of this study were therefore to further define the interkingdom
288 interactions of these two microbes and their capacity to modulate GU tract colonisation,
289 an essential step in the pathogenesis of both microorganisms.

290 Using VEC line VK2/E6E7 as a model system, this study provides evidence that a
291 reciprocal, synergistic relationship exists between GBS and *C. albicans* that may serve
292 to promote their co-colonisation of the vaginal mucosa. Specifically, when incubated
293 together, numbers of both microbes associated with the epithelial monolayers were
294 found to be significantly higher than the numbers recovered from equivalent

295 monospecies samples. Confocal microscopy revealed extensive hyphal ‘mats’ of
296 candidal cells overlaying the epithelial monolayers to which GBS cells were attached.
297 This infers that direct physical contact (i.e. coadhesion) between GBS and *C. albicans* is
298 a key mechanism that contributes to their synergistic interplay. Thus GBS may bind
299 directly to epithelium or to adherent *C. albicans* cells, and vice versa.

300 To identify the mechanistic basis of coadhesion between GBS and *C. albicans*,
301 studies focused on the hypha-specific adhesins of *C. albicans* and specifically adhesin
302 Als3, since a distinct tropism for candidal hyphae was observed for both GBS strains
303 tested. Use of Als3 knockout and complemented strains of *C. albicans* confirmed that
304 recognition of this glycoprotein by GBS is required for effective coaggregation of these
305 two microbes under planktonic conditions, and for co-association with vaginal
306 epithelium. This correlates well with the interactions of *C. albicans* and streptococci
307 within the oral cavity reported to date (32, 34) and thus may infer that Als3 recognition
308 represents a common mechanism for *C. albicans* engagement by the *Streptococcus*
309 genus. The addition of GBS to the list of microbes that utilise Als3 as a receptor,
310 alongside other streptococci, *Staph. aureus* and *Rothia dentocariosa* (32, 37, 43), also
311 adds support to the notion that Als3 plays a major role in the capacity for *C. albicans* to
312 mediate a diverse range of polymicrobial interactions.

313 In addressing the GBS side of this synergistic partnership, this study provides
314 evidence for the role of GBS AgI/II family (Bsp) adhesins in this process. Previous work
315 has implicated BspA in facilitating coaggregation of GBS strain NEM316 with *C.*
316 *albicans* under planktonic conditions (12). These data are supported here and
317 developed to include adhesin BspC, implying that these capabilities may represent

318 functions that are shared across the Bsp adhesin family. Moreover, loss of BspC
319 impaired GBS co-association with *C. albicans*, while expression of BspC by *L. lactis*
320 enabled *C. albicans* to promote association of this surrogate host with VEC monolayers.
321 This extends our current understanding of the properties of the adhesin family and
322 implies that Bsp adhesins are determinants of GBS that facilitate co-association with *C.*
323 *albicans* on vaginal epithelium. Moreover, coggregation between surrogate hosts
324 expressing Als3 and Bsp adhesins adds support to the hypothesis that direct binding
325 between Bsp polypeptides of GBS and Als3 of *C. albicans* is a mechanism that
326 underpins, at least in part, the synergy in epithelial cell interactions between these two
327 microbes. Interestingly, while deletion of *bspC* did not ablate co-association between
328 GBS and *C. albicans*, deletion of both *als3* alleles effectively prevented the interaction.
329 This indicates a role for additional GBS determinants in mediating the interkingdom
330 relationship, and implies that these determinants may also target candidal receptor
331 Als3. This supports the evidence that Als3 has capacity to bind multiple, diverse ligands
332 (44).

333 Based on primary sequence, the AgI/II family polypeptides of GBS can be divided
334 into four homologues: BspA and B, which share 90% sequence identity, and BspC and
335 D, which share 99% sequence identity (12). The highest level of variation between
336 BspA/B and BspC/D is seen within the N-terminal alanine-rich and proline-rich domains.
337 By contrast, the V domain shares 96-100% sequence identity across all four Bsp
338 homologues (12). The V domain has been identified as the functional region of a
339 number of AgI/II family polypeptides (45-47), including BspA, where it was shown to
340 promote binding of GBS NEM316 to scavenger receptor agglutinin glycoprotein-340

341 (12). If the V domain is also responsible for GBS co-association with *C. albicans*, the
342 high level of sequence similarity may explain why both BspA and BspC display
343 comparable functional properties. Delineating the precise domains within Bsp that are
344 required for engagement with candidal Als3 will be the focus of future studies.

345 It is clear that direct physical contact between *C. albicans* and GBS plays a
346 significant role in their co-association with VECs. We also considered the possibility that
347 intermicrobial signals played a role in the processes described. However, no evidence
348 was found for diffusible molecules released by either *C. albicans* or GBS having the
349 capacity to significantly modulate microbial interactions with vaginal epithelium.
350 Nonetheless, provision of additional intermicrobial binding sites may not be the only
351 mechanism involved in the synergy with VECs. For example, in dual-species images
352 there were patches of epithelium that were heavily colonised by GBS while seemingly
353 devoid of *C. albicans* (Fig. 3). Fixation of VECs did not inhibit co-association between
354 GBS and *C. albicans*, implying that these effects are not dependent upon modulation of
355 epithelial cell biology (e.g. receptor availability). Nonetheless, it remains possible that
356 GBS engagement with *C. albicans* alters the GBS receptor profile such that these
357 bacteria are subsequently more permissive to interactions with VECs. The large impact
358 of *als3* gene deletion on the GBS-*C. albicans*-VEC co-association raises the prospect
359 that Als3 may mediate such effects. Future studies will explore these possible
360 explanations.

361 To conclude, this study identifies for the first time a synergistic interplay between
362 GBS and *C. albicans* that enhances the capacity of both microorganisms to associate
363 with vaginal epithelial cells. Molecular determinants critical to this co-association

364 mechanism are identified as Bsp adhesins of GBS, and Als3 of *C. albicans*. GU tract
365 colonisation is an essential first step in the pathogenesis of diseases such as vaginal
366 thrush, and is a significant risk factor for GBS neonatal disease due to vertical
367 transmission. Co-association between GBS and *C. albicans* may therefore have
368 important implications for disease risk by both of these opportunistic pathogens. This
369 co-association also raises the intriguing possibility of utilising a convergent immunity
370 approach to develop novel intervention strategies, as has been explored for *C. albicans*
371 and *Staph. aureus* (48). There is currently no vaccine against GBS disease.
372 Furthermore, while use of intrapartum antibiotic prophylaxis (IAP) has been successful
373 in decreasing the incidence of early-onset neonatal GBS disease in some countries, the
374 logisitics of IAP make it an unrealistic control strategy for rural and developing countries,
375 and IAP has had no impact on the rate of late-onset GBS infection (49, 50). The data
376 presented here imply that better control of vaginal colonisation by *C. albicans* may
377 restrict or reduce GBS colonisation, which in turn would reduce the risk of GBS
378 transmission. Hence vaccines against *C. albicans*, such as the promising rAls3 vaccine
379 that has completed phase 1 clinical trials (51), could concomitantly help to reduce the
380 burden of neonatal GBS disease.
381
382 **MATERIALS AND METHODS**
383 **Microbial strains and culture conditions.** The microbial strains used in this
384 study are listed in Table 1. GBS strains were cultured in Todd-Hewitt broth with 0.5%
385 Yeast Extract (THY) or on THY agar plates at 37 °C, 5% CO_2. *Lactococcus lactis* was
386 cultured in GM17 broth (M17 broth supplemented with 0.5% glucose) or on GM17 agar

387 plates at 30 °C in a candle jar. *Escherichia coli* was cultured aerobically in Luria Bertani
388 (LB) broth or on LB agar plates at 37 °C. Media were supplemented with 5 µg/ml
389 erythromycin, or with 50 µg/ml (*E. coli*) or 5 µg/ml (GBS) chloramphenicol as
390 appropriate. Heterologous protein expression in *L. lactis* was induced from nisin-
391 inducible plasmids by the addition of 10 ng/ml nisin. Cells from GBS and *L. lactis* broth
392 cultures were harvested by centrifugation at 5000 *g* for 7 min.
393 *C. albicans* was cultured in YPD medium (1% Yeast Extract, 2% Mycological
394 peptone, 2% glucose) at 37 °C with shaking (220 rpm), or maintained on Sabouraud
395 dextrose (SAB) agar plates incubated aerobically at 37 °C. *C. albicans* cells were
396 harvested from broth cultures by centrifugation at 5000 *g* for 5 min. *S. cerevisiae* was
397 cultured in complete supplement medium (CSM) without uracil (ForMedium)
398 supplemented with 0.67% yeast nitrogen base (YNB; Difco) and 2% glucose at 30 ^{o}C
399 with shaking.
400 **Generation of GBS knockout and *L. lactis* surrogate expression strains.** A
401 Δ*bspC* mutant was generated in GBS strain 515 by in-frame allelic replacement with a
402 chloramphenicol resistance cassette by homologous recombination, using a method
403 previously described (52). Briefly, a knockout construct was generated by amplifying
404 flanking regions directly upstream and downstream of the *bspC* gene from GBS strain
405 515 genomic DNA using primer pairs *bspC*.F1/*bspC*.R1 and *bspC*.F2/*bspC*.R2
406 respectively (Table 2). A *cat* cassette was amplified from chloramphenicol-resistant
407 plasmid pR326 using primers *cat*.F and *cat*.R (Table 2). Upstream and downstream
408 *bspC* and *cat* amplicons were then combined by stitch PCR using primers *bspC*.F1 and
409 *bspC*.R2. The resultant amplicon was cloned into vector pHY304 (53) via *Xba*I and

410 *Bam*HI sites, and propagated in *E. coli* Stellar^{TM} (Clontech) prior to isolation and
411 electroporation into GBS 515.
412 A *L. lactis* strain expressing BspA had been generated previously (12), and a
413 similar methodology was employed here to generate a *L. lactis* strain expressing BspC.
414 In brief, the *bspC* gene was amplified from GBS strain 515 genomic DNA using primers
415 pMSP.*bspC*.F and pMSP.*bspC*.R (Table 2). The resultant amplicon was then cloned
416 into nisin-inducible expression vector pMSP7517 (54) via *Nco*I and *Xho*I sites,
417 generating plasmid pMSP.*bspC*. This construct was transformed directly into
418 electrocompetent *L. lactis* NZ9800, as described previously (12). Transformants were
419 confirmed by plasmid isolation and PCR, while expression of BspC in *L. lactis* was
420 verified by dot immunoblot.
421 **Tissue culture.** Experiments were conducted using VK2/E6E7 cells (ATCC CRL-
422 2616), an immortalised human VEC line with a similar protein profile to the natural
423 tissue (55, 56). VECs were cultured in K-SFM (Gibco®) supplemented with 0.4 mM
424 $CaCl_2$, 0.05 mg/ml Bovine Pituitary Extract and 0.1 ng/ml Epidermal Growth Factor.
425 Upon reaching 70-80% confluence, cells were disassociated using TrypLE express
426 trypsin replacement reagent (Gibco®) before being harvested and resuspended in K-
427 SFM. Appropriate volumes of cells were seeded to fresh flasks or to assay plates, as
428 required.
429 **Visualisation of dual-species planktonic interactions.** Cells from 16 h cultures
430 of *C. albicans* were harvested, washed in YNBPT (1x Yeast Nitrogen Base, 20 mM
431 Na_2HPO_4, 0.02% tryptone, adjusted to pH 7) and suspended to OD_{600} 1.0 (equivalent to
432 10^6 cells/ml) in YNBPT. This suspension was diluted 1:10 into YNBPTG (YNBPT

433 supplemented with 0.4% glucose) and incubated at 37 °C, 220 rpm for 2 h (2 ml final
434 volume). These growth conditions have previously been shown to induce candidal
435 hyphae formation (57).

436 GBS cells were harvested from 16 h cultures, washed in YNBPT, and then
437 suspended in 1.5 mM fluorescein isothiocyanate (FITC) dissolved in carbonate buffer
438 (100 mM NaCl, 50 mM Na_2CO_3), and incubated for 30 min with gentle agitation. GBS
439 cells were harvested, washed three times in carbonate buffer and the pellet suspended
440 and adjusted to OD_{600} 0.5 (equivalent to $5x10^7$ cells/ml) in YNBPTG. GBS suspension
441 (1 ml) was added to that of *C. albicans* and incubated at 37 °C for a further 1 h with
442 shaking. Calcofluor White (0.00001% in dH_2O) was added before visualisation of 10 µl
443 samples by fluorescence microscopy.

444 For quantification assays, approximately 40 images were taken of randomly
445 selected hyphae from each experimental group. Interactions were scored into one of
446 four groups, similar to the method reported previously (32): 0 interacting bacteria per
447 hypha, 1-5 bacteria, 6-20 bacteria, and more than 20 bacteria per hypha.

448 In a variation of this assay, *Saccharomyces cerevisiae* cells were harvested from a
449 16 h overnight broth culture in CSM broth, washed once in YNBPT (5 ml), and stained
450 in 1.5 mM FITC for 30 min with gentle agitation. *S. cerevisiae* were harvested and
451 washed three times in carbonate buffer. The pellet was suspended and adjusted to
452 OD_{600} 1.0 (equivalent to 10^6 cells/ml) in YNBPTG, before 1:5 dilution into YNBPTG (final
453 volume 2 ml). This suspension was incubated at 30 °C, 220 rpm for 3 h. *L. lactis* strains
454 were harvested from a 16 h overnight broth culture and washed once in YNBPT before
455 suspension in 2 mL TRITC (0.1 mg/ml in carbonate buffer) and incubated for 30 min

456 with gentle agitation. *L. lactis* cells were harvested, washed three times in carbonate
457 buffer and adjusted to OD_{600} 0.5 in YNBPTG (equivalent to $5x10^7$ cells/ml). Adjusted *L.*
458 *lactis* suspension (1 ml) was added to *S. cerevisiae* and incubated for a further 1 h at 30
459 °C, 220 rpm, before visualisation of 10 µl samples by fluorescence microscopy.

460 **Microbial growth in dual-species broth cultures.** Cells from overnight (16 h) *C.*
461 *albicans* suspension culture were harvested and washed once in phosphate-buffered
462 saline (PBS). The pellet was suspended and adjusted to OD_{600} 1.0 in K-SFM before a
463 1:10 dilution into K-SFM (2 ml final volume) and incubation at 37 °C, 220 rpm for 2 h.
464 Cells from overnight GBS broth cultures were harvested, washed once in PBS, and
465 suspended in K-SFM at OD_{600} 0.5. GBS suspension (1 ml) was added to *C. albicans*
466 suspension and the mixture incubated at 37 °C for a further 1 h. Planktonic suspensions
467 were vortex mixed for 15 s before serial 10-fold dilutions into THY broth. Numbers of
468 microorganisms were detected by viable count (CFU) on either THY agar plates (GBS)
469 supplemented with 50 µg/ml nystatin to inhibit *C. albicans* growth, or SAB agar plates
470 (*C. albicans*) supplemented with 5 µg/ml erythromycin to inhibit GBS growth.

471 **Epithelial association assay.** Epithelial association assays were conducted as
472 described by (5) with a few modifications. VECs were seeded into a 24-well plate at
473 $2x10^5$ cells/well and incubated until confluent at 37 °C, 5% CO_2 (48-72 h). *C. albicans*
474 cells were diluted in K-SFM to obtain approximately $5x10^5$ cells/ml, while GBS or *L.*
475 *lactis* cells were diluted into K-SFM to obtain approximately $5x10^5$ cells/ml.

476 Wells containing VEC monolayers were washed once with PBS and
477 approximately $5x10^5$ bacteria or *C. albicans* (1 ml; MOI 2.5) were then added to each
478 well. Bacterial suspensions were incubated at 37 °C, 5% CO_2 for 1 h, while *C. albicans*

479 suspensions were incubated for 2 h. For dual species assays, *C. albicans* suspensions
480 were incubated for 1 h before the medium was replaced by GBS or *L. lactis* and
481 incubated for a further 1 h. For all assays, wells were then washed three times with PBS
482 before incubation for 15 min with TrypLE™, followed by two ice cold water incubations,
483 lasting 20 min each, to lyse the VECs. Lysates were serially diluted onto THY (GBS),
484 GM17 (*L. lactis*) or SAB (C*. albicans*) agar plates and viable counts determined, as
485 described above. It was confirmed both visually and by monitoring levels of lactate
486 dehydrogenase (LDH) released into the culture supernatants that epithelial monolayers
487 remained intact and viable over the time periods for mono- or dual-species association
488 assays.
489 In a variation of this assay, VEC monolayers were fixed in 2% paraformaldehyde
490 overnight prior to incubation with cell suspensions of *C. albicans* and/or GBS.
491 Alternatively, GBS suspensions were prepared as described above and preincubated at
492 room temperature with 10 µg/ml rabbit preimmune or anti-Bsp sera (Eurogentec) for 30
493 min, prior to incubation at 37 ^{o}C for 1 h with VEC monolayers.
494 **Spent media studies.** VECs were seeded in a 24-well plate and grown to
495 confluence. *C. albicans* was prepared as described above, and incubated with the
496 VECs, or grown planktonically in K-SFM medium, for 1 h. *C. albicans* media were then
497 collected and sterilised by filtration through a 0.2 µm filter. GBS suspensions, prepared
498 as above, were adjusted to OD_{600} 1.0 in K-SFM, before a 1:200 dilution into either fresh
499 K-SFM, K-SFM from *C. albicans* planktonic growth, or K-SFM from *C. albicans* growth
500 on VK2/E6E7 monolayers. Aliquots (1 ml) were added to VEC monolayers and

501 incubated for 1 h. VECs were disassociated and lysed as above, with CFU of GBS
502 determined by serial dilution and viable count on THY agar plates.
503 **Transwell studies.** VECs were seeded in a 24-well plate and grown to
504 confluence. *C. albicans* cells were prepared as above, and incubated with VEC
505 monolayers for 1 h before the medium was replaced with 1 ml K-SFM. Transwell inserts
506 with high density pores of 0.4 μm (Sarstedt) were placed into wells. GBS suspensions in
507 K-SFM (OD_{600} 1.0) were diluted 1:100 into K-SFM. Aliquots (0.5 ml) were added to the
508 transwell inserts and the plates incubated for a further 1 h. The inserts were removed,
509 remaining VECs were disassociated and lysed as described above, and *C. albicans*
510 CFU determined by serial dilution and viable count on SAB agar plates.
511 **Confocal microscopy.** For visualisation by confocal microscopy, VEC
512 monolayers were grown on 19 mm glass cover slips in a 12-well plate until confluent.
513 The epithelial association assay was then carried out as described above, except that
514 the time was extended by 4 h. Calcofluor White (1 μl) was added to stain the chitin in
515 the *C. albicans* cell wall, and the cover slips then fixed in 2% paraformaldehyde. Triton
516 X-100 (0.3%) was used to permeabilise the epithelial cells before blocking in 2% BSA.
517 Bacteria were stained with a mouse anti-GBS antibody (1.B.501, Santa Cruz
518 Biotechnology) followed by a goat anti-mouse Alexafluor-488 conjugated antibody
519 (Fisher), both of which were used at a dilution of 1:200. The F-actin of the epithelial cells
520 was stained with phalloidin-TRITC (Sigma). Cover slips were then mounted onto glass
521 slides using Vectashield (Vector Labs), and imaged on a Leica SP5-AOBS confocal
522 laser scanning microscope (CSLM) attached to a Leica DM I6000 inverted
523 epifluorescence microscope. Images were processed using Volocity® software and

524 Imaris® v7.5 software (Bitplane AG, Zurich, Switzerland) was used to calculate
525 biovolumes (μm^3).

526 **Statistical analyses.** All assays were performed in triplicate unless otherwise
527 stated. Data were analysed using unpaired Student's *t* tests with Bonferroni correction,
528 as appropriate.

529

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537

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716

717 **FIGURE LEGENDS**

718 **FIG 1** Fluorescence micrographs of planktonic interactions between *C. albicans* and
719 GBS. *C. albicans* SC5314 was grown in YNBPTG for 2 h at 37 °C, 220 rpm before
720 addition of (A) GBS strain NEM316 or (B) GBS strain 515, and incubation for a further 1
721 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor
722 White (blue). Scale bars, 20 μm.

723

724 **FIG 2** Effects of *C. albicans* SC5314 on association of GBS with VECs. VEC
725 monolayers were incubated with GBS suspensions (MOI 2.5) for 1 h (open bars) or with
726 *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars). Monolayers
727 were then lysed and numbers of associated GBS enumerated by serial dilution onto

728 THY agar supplemented with 50 µg/mL nystatin. * indicates $P<0.05$ compared to

729 monospecies controls, as determined by unpaired Student's *t*-test; n = 4.

730

731 **FIG 3** Representative confocal micrographs of *C. albicans*-GBS association with VECs.

732 VEC monolayers were incubated with GBS alone for 5 h (columns 1 and 2) or with *C.*

733 *albicans* for 1 h followed by GBS for a further 5 h (columns 3 and 4). Cells were then

734 fixed, stained and mounted onto glass slides. GBS was labelled using Alexafluor-488-

735 conjugated antibody (green), while *C. albicans* was labelled with Calcofluor White

736 (blue), and VECs were labelled with phalloidin-TRITC (red). GBS strains NEM316 (top

737 panels) and 515 (bottom panels) were tested. Columns 2 and 4 are duplicates of

738 columns 1 and 3, respectively, in which the red filter (i.e. the VECs) has been removed

739 (VK2/E6E7 off). Scale bars, 100 µm. White arrows indicate areas where GBS binds *C.*

740 *albicans* hyphae, while red arrows indicate areas where GBS is found in the absence of

741 *C. albicans*.

742

743 **FIG 4** Effects of *C. albicans* on association of GBS with VECs following 5 h incubation.

744 (A) Quantification of GBS from confocal micrographs illustrated in Fig. 3. Images were

745 processed using Volocity® software and Imaris® software was used to calculate GBS

746 biovolumes (μm^3). (B) Quantification of GBS by viable count. VEC monolayers were

747 incubated with GBS suspensions for 5 h (open bars) or with *C. albicans* for 1 h followed

748 by GBS for a further 5 h (black bars). Monolayers were then lysed and numbers of

749 associated GBS enumerated by serial dilution onto THY agar supplemented with 50

750 μg/mL nystatin. ** indicates *P*<0.01 compared to monospecies controls, as determined
751 by unpaired Student's *t*-test; n = 4.
752
753 **FIG 5** Effects of GBS on association of *C. albicans* SC5314 (WT) with VECs. VEC
754 monolayers were incubated with *C. albicans* cells for 1 h to allow production of hyphae.
755 GBS suspensions were then added for a further 1 h, before monolayers were lysed.
756 Numbers of associated *C. albicans* were enumerated by serial dilution onto SAB agar
757 supplemented with 5 μg/mL erythromycin. * indicates *P*<0.05 compared to the
758 monospecies control, as determined by unpaired Student's *t*-test with Bonferroni
759 correction; n = 3.
760
761 **FIG 6** Growth of *C. albicans* or GBS in mono- or dual-species suspension culture. K-
762 SFM broth cultures were inoculated with *C. albicans* SC5314 (WT) at 37 °C, 220 rpm for
763 1 h before addition of GBS and incubation for a further 1 h (black bars). Alternatively
764 broth cultures were inoculated with *C. albicans* or GBS alone and incubated for 2 h or 1
765 h respectively (open bars). *C. albicans* CFU/mL were then determined by viable count
766 onto SAB agar supplemented with 5 μg/mL erythromycin (A), while GBS CFU/mL were
767 determined by viable count onto THY agar supplemented with 50 μg/mL nystatin (B).
768 'NS' indicates *P*>0.05 compared to the monospecies control, as determined by unpaired
769 Student's *t*-test; n = 3.
770
771 **FIG 7** Role of contact-independent mechanisms or fixation in modulating interactions of
772 *C. albicans* or GBS with VECs. (A) *C. albicans* SC5314 (WT) was grown on VEC

773 monolayers, or planktonically in K-SFM medium, for 1 h before spent media were
774 collected and filter sterilised. GBS cells were incubated in these spent media on VECs
775 for 1 h, before monolayers were lysed and numbers of associated GBS enumerated by
776 serial dilution onto THY agar. 'NS' indicates $P>0.05$ compared to the blank K-SFM
777 control, as determined by unpaired Student's *t*-test; n = 3. (B) *C. albicans* was grown on
778 VEC monolayers for 1 h, before GBS suspensions or K-SFM alone were placed into
779 transwell baskets suspended above. After a further 1 h incubation, *C. albicans* was
780 enumerated by serial dilution onto SAB agar. (C,D) VEC monolayers were fixed with 2%
781 paraformaldehyde and then incubated with GBS suspensions (MOI 2.5) for 1 h (open
782 bars) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars).
783 Monolayers were then lysed and GBS CFU/mL determined by viable count onto THY
784 agar supplemented with 50 µg/mL nystatin (C), while *C. albicans* CFU/mL were
785 determined by viable count onto SAB agar supplemented with 5 µg/mL erythromycin
786 (D). 'NS' indicates $P>0.05$, ** indicates $P<0.01$ compared to monospecies controls, as
787 determined by unpaired Student's *t*-test with Bonferroni correction; n = 4 (A,B) or 3
788 (C,D).
789
790 **FIG 8** Effects of *C. albicans* or Bsp antisera on the association of GBS wild type or
791 isogenic mutant strains with VECs. (A) VEC monolayers were incubated with GBS wild
792 type (WT) 515 or mutant Δ*bspC* cell suspensions (MOI 2.5) for 1 h (open bars), or with
793 *C. albicans* SC5314 (MOI 2.5) for 1 h followed by addition of strain 515 suspensions for
794 a further 1 h (black bars). Monolayers were then lysed and numbers of associated GBS
795 were enumerated by serial dilution onto THY agar supplemented with 50 µg/mL

796 nystatin. ** indicates significance relative to monospecies controls; Ω indicates
797 significance relative to wild type monospecies control; § indicates significance relative to
798 wild type in the presence of *C. albicans*. (B) GBS cell suspensions were preincubated
799 with preimmune (open bars) or anti-Bsp (black bars) sera, before incubation with VEC
800 monolayers (MOI 2.5) for 1 h and enumeration from cell lysates by viable count. **
801 indicates significance relative to preimmune controls. Significance indicates *P*<0.01, as
802 determined by unpaired Student's *t*-test with Bonferroni correction; n = 3.
803
804 **FIG 9** Effects of *C. albicans* on the association of *L. lactis* Bsp surrogate expression
805 strains with VECs. VEC monolayers were incubated with suspensions of *L. lactis* pMSP
806 vector control, pMSP.BspA or pMSP.BspC (MOI 2.5) for 1 h (open bars), or with *C.*
807 *albicans* SC5314 (MOI 2.5) for 1 h followed by addition of *L. lactis* suspensions for a
808 further 1 h (black bars). Monolayers were then lysed and numbers of associated *L.*
809 *lactis* were enumerated by serial dilution onto GM17 agar supplemented with 50 μg/mL
810 nystatin. ** indicates significance relative to monospecies controls; Ω indicates
811 significance relative to pMSP empty vector control; § indicates significance relative to
812 pMSP empty vector control in the presence of *C. albicans*. Significance indicates
813 *P*<0.01, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 4.
814
815 **FIG 10** Role of Als3 in planktonic interactions between *C. albicans* and GBS. (A)
816 Fluorescence micrographs of planktonic interactions between *C. albicans* Δ*als3* (left
817 panel) or Δ*als3*+*als3* complemented strain (right panel) with GBS strains NEM316 (top
818 panels) or 515 (bottom panels). *C. albicans* was grown in YNBPTG for 2 h at 37 °C, 220

819 rpm before addition of GBS and incubation for a further 1 h. GBS was labelled with
820 FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars,
821 20 µm. Note, interactions of GBS strains with *C. albicans* SC5314 wild type are shown
822 in Fig. 1. (B) Semi-quantitation of numbers of *C. albicans* hyphae with 0, 1-5, 6-20 or
823 >20 interacting GBS, based on approximately 40 randomly selected images from each
824 experimental group. * indicates $P<0.05$; 'NS' indicates $P>0.05$, as determined by linear
825 regression analysis of datasets; n = 4.
826
827 **FIG 11** Role of Als3 in synergistic effects of *C. albicans* on association of GBS with
828 VECs. VEC monolayers were incubated with GBS suspensions for 1 h (open bars) or
829 with *C. albicans* SC5314 wild type (WT) (black bars), Δ*als3* (grey bars) or Δ*als3*+*als3*
830 (hashed bars) for 1 h, followed by GBS for a further 1 h. Monolayers were lysed and
831 then numbers of associated GBS were enumerated by serial dilution onto THY agar
832 supplemented with 50 µg/mL nystatin. ** indicates $P<0.01$; 'NS' indicates $P>0.05$, as
833 determined by unpaired Student's *t*-test with Bonferroni correction; n = 4.
834
835 **FIG 12** Role of Als3 in synergistic effects of GBS on association of *C. albicans* with
836 VECs. VEC monolayers were incubated with *C. albicans* SC5314 wild type (WT) (open
837 bars), Δ*als3* (black bars) or Δ*als3*+*als3* (striped bars) for 1 h followed by GBS for a
838 further 1 h. Monolayers were lysed and numbers of associated *C. albicans* were
839 enumerated by serial dilution onto SAB agar supplemented with 5 µg/mL erythromycin.
840 ** indicates $P<0.01$; 'NS' indicates $P>0.05$, as determined by unpaired Student's *t*-test
841 with Bonferroni correction; n = 4.

842

843 **FIG 13** Fluorescence micrographs of planktonic interactions between *S. cerevisiae* Als3
844 and *L. lactis* Bsp surrogate expression strains. *S. cerevisiae* (Als3+) was grown in
845 YNBPTG for 3 h at 30 °C, 220 rpm before addition of (A) *L. lactis* (pMSP control), (B) *L.*
846 *lactis* (pMSP.BspA) or (C) *L. lactis* (pMSP.BspC) and incubation for a further 1 h. *L.*
847 *lactis* was labelled with TRITC (red), while *S. cerevisiae* was labelled with FITC (green).
848 Scale bars, 20 μm.

849 **TABLE 1** Microbial strains used in this study

Strain or plasmid	Unique ID #	Relevant genotype	Reference or source
C. albicans			
SC5314	UB1843	Wild type	Neil Gow, Univ Aberdeen
	UB1941	Δ*als3*	(39)
	UB1940	Δ*als3*+pUL.*als3*	(39)
S. cerevisiae			
BY4742	UB2156	pBC542-*ALS3sm*	(40)
S. agalactiae			
NEM316	UB1931	Wild type	Shaynoor Dramsi, Institut Pasteur
515	UB2410	Wild type	Victor Nizet, Univ California San Diego
515	UB2873	Δ*bspC*	This study
L. lactis			
NZ9800	UB2635	pMSP	(12)
	UB2658	pMSP.*bspA*	(12)
	UB2659	pMSP.*bspC*	This study
Plasmids			
pMSP7517		*E.coli*-enterococcal shuttle vector	(54)

	containing *E. faecalis* *prgB* under control of *nisA* promoter; erythromycin[R]	
pMSP.*bspC*	pMSP7517-derived containing *bspC* from GBS 515 in place of *prgB*	
pR326	chloramphenicol[R]	(58)

850

851

852

853

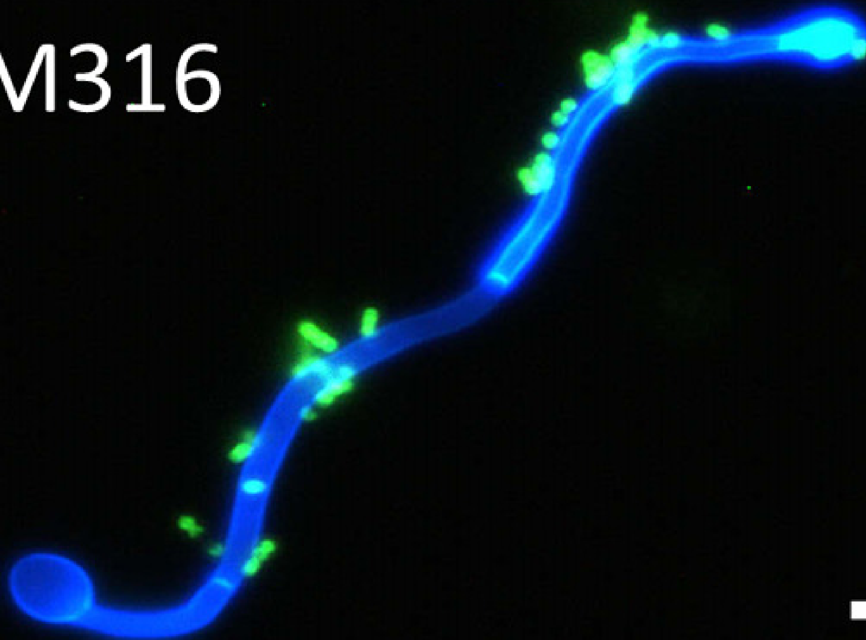
854 **TABLE 2** Primers used in this study

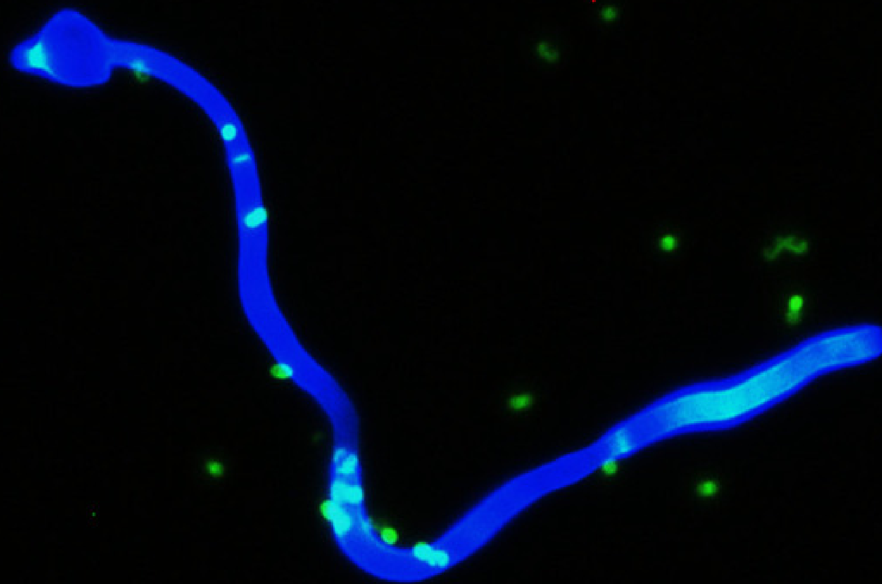
Primer name	Sequence[a]
bspC.F1	GCTCTAGAGCAATTAGCAGATGCACAG
bspC.R1	TAAAATCAAAGGAGAAAATATGAACTTTA
bspC.F2	GCTTTTATAATCAATATTCAGAAGCACTTG
bspC.R2	CGGGATCCGAGCCAAATTACCCCTCC
cat.F	AGAAAATATGAACTTTAATAAAATTGATTTAG
cat.R	TGAATATTGATTATAAAAGCCAGTCATTAGG
pMSP.*bspC*.F	CATGCCATGGAGGAGGAAATATGTATAAAAATCAAAAC
pMSP.*bspC*.R	CCGCTCGAGGCAGGTCCAGCTTCAAATC

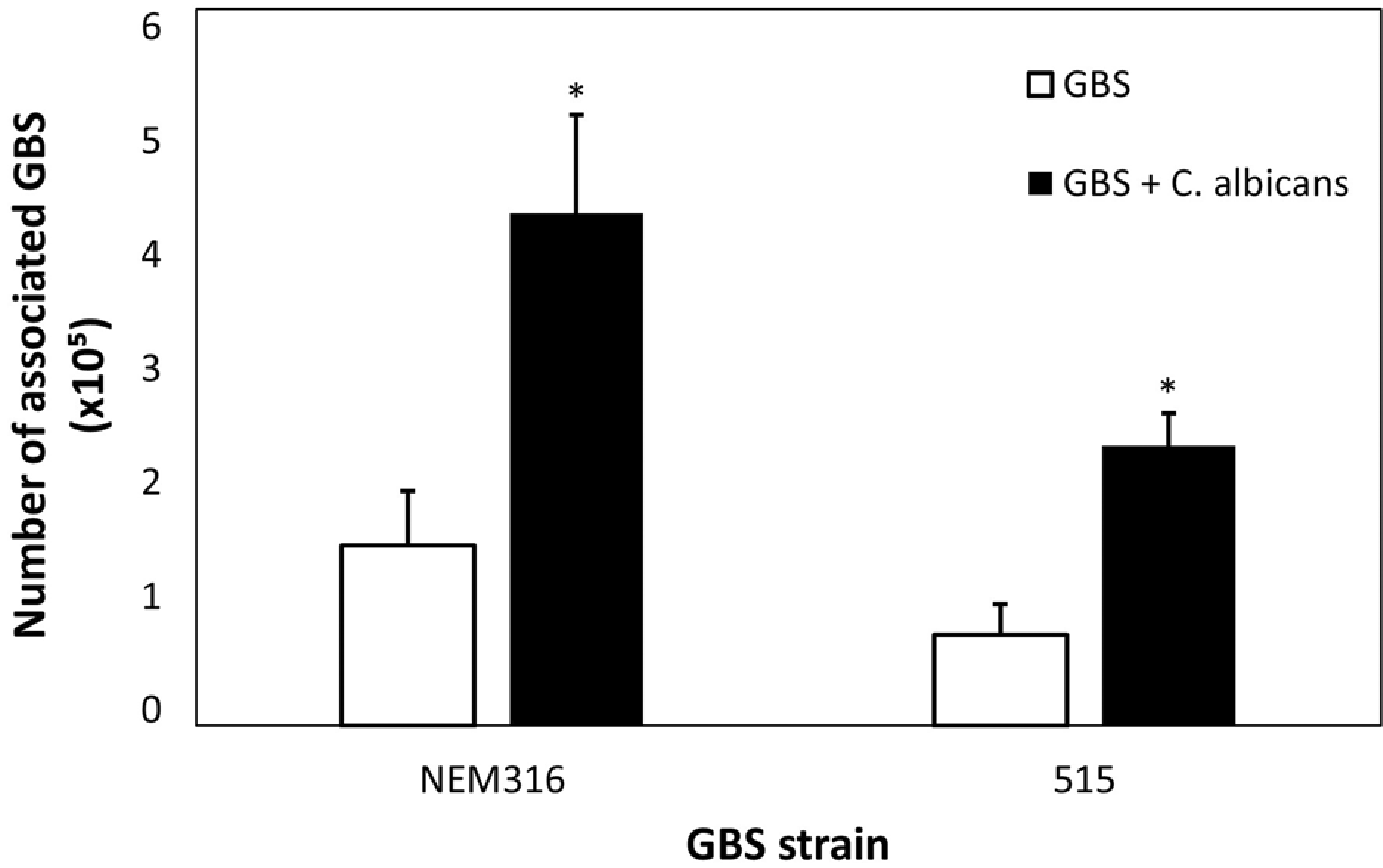
855

856 [a] Restriction endonuclease sites are underlined.

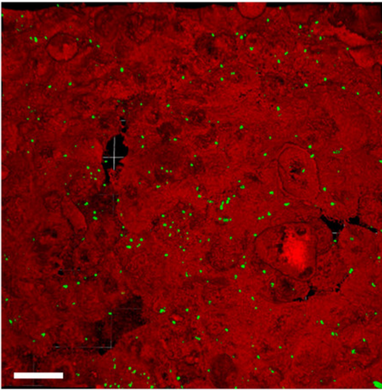
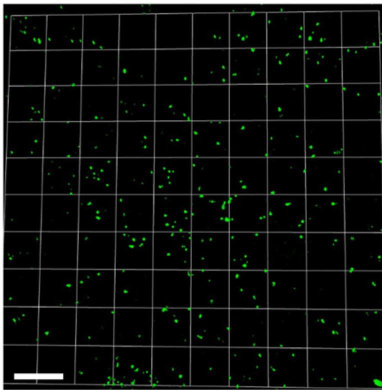
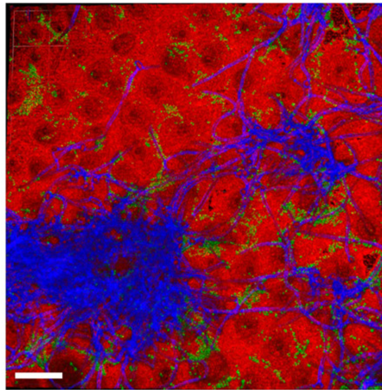
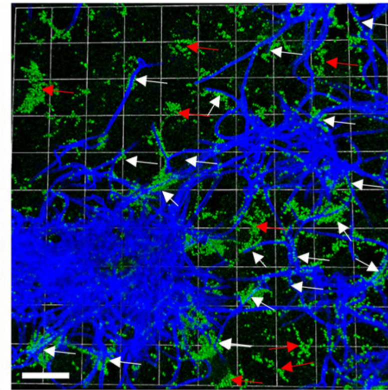
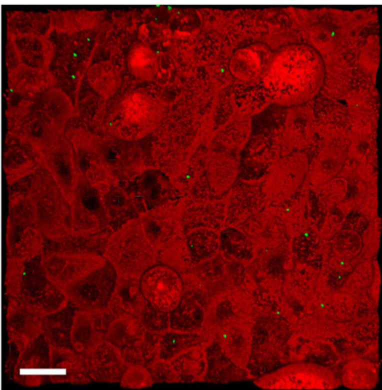
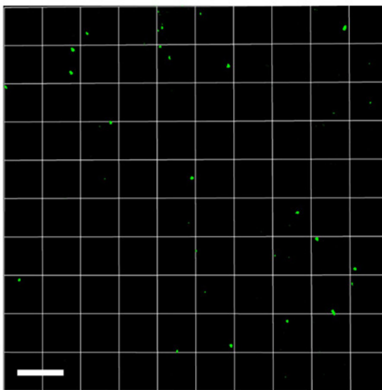
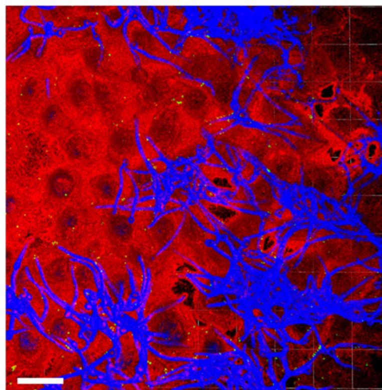
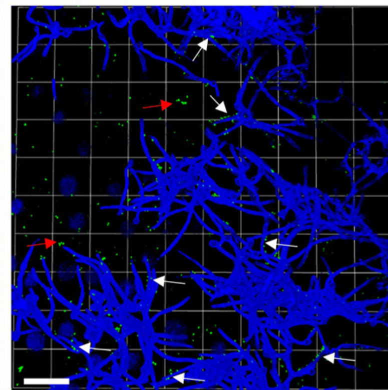
857


A) NEM316


B) 515



Number of associated GBS (x10⁵)
6
5
4
3
2
1
0
GBS
GBS + C. albicans
*
*
NEM316
515
GBS strain

	VK2/E6E7+GBS	VK2/E6E7+GBS (VK2/E6E7 off)	VK2/E6E7+GBS+ *C. albicans*	GBS+*C. albicans* (VK2/E6E7 off)
NEM316				
515				

A

3,500
3,000
2,500
2,000
1,500
1,000
500
0

Biovolume (µm³)

**
**

GBS
GBS+C. albicans

NEM316
515

B

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

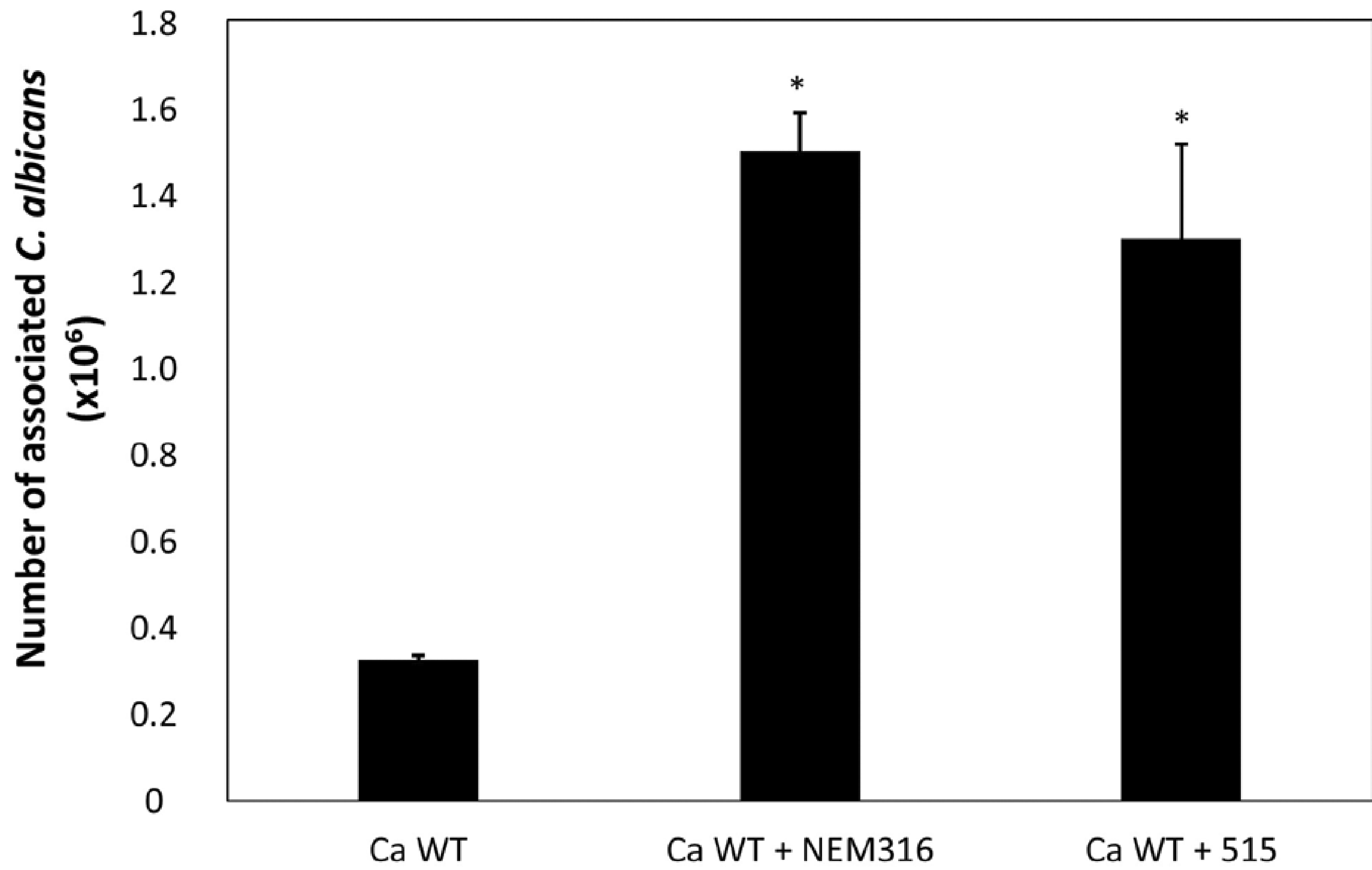
Number of associated GBS (x10⁸)

**
**

GBS
GBS+C. albicans

NEM316
515

GBS strain



Number of associated C. albicans (x10^6)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
Ca WT
Ca WT + NEM316
Ca WT + 515

A

Number of *C. albicans* (x10⁵)

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

NS
NS

Ca WT
Ca WT + NEM316
Ca WT + 515

B

Number of GBS (x10⁸)

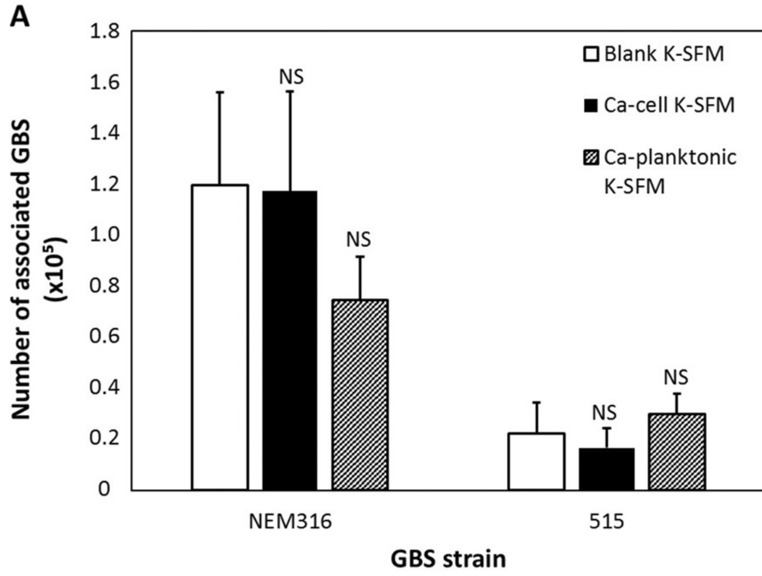
7
6
5
4
3
2
1
0

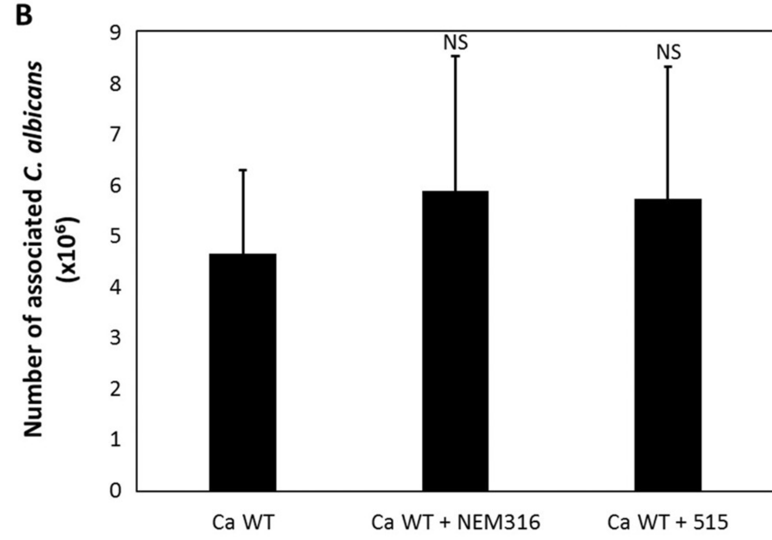
GBS
GBS + C. albicans

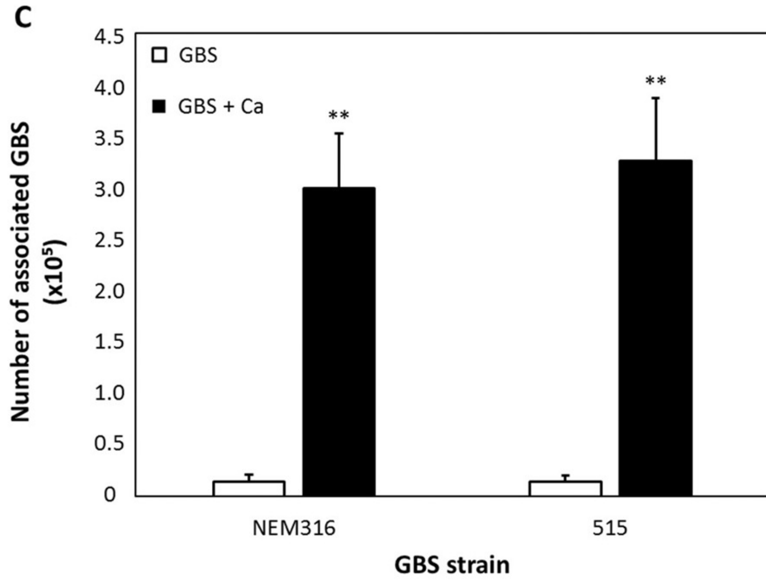
NS
NS

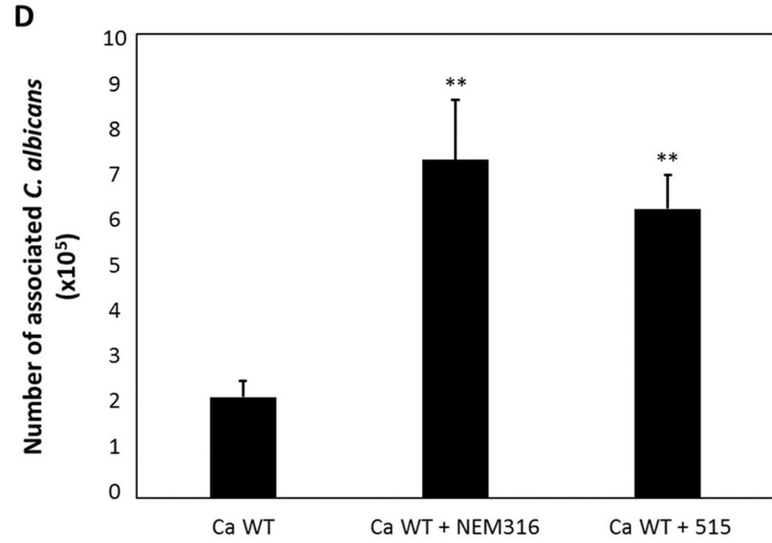
NEM316
515

GBS strain


A
Number of associated GBS (x10⁵)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Blank K-SFM
Ca-cell K-SFM
Ca-planktonic K-SFM
NS
NS
NS
NS
NEM316
515
GBS strain


B
Number of associated C. albicans (x10⁶)
9
8
7
6
5
4
3
2
1
0
NS
NS
Ca WT
Ca WT + NEM316
Ca WT + 515


C
Number of associated GBS (x10⁵)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GBS
GBS + Ca
**
**
NEM316
515
GBS strain


D
Number of associated C. albicans (x10⁵)
10
9
8
7
6
5
4
3
2
1
0
**
**
Ca WT
Ca WT + NEM316
Ca WT + 515

A

Number of associated GBS ($x10^5$)

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0

GBS

GBS+C. albicans

**

§

**

Ω

515 WT

515 ΔbspC

GBS strain

B

Number of associated GBS ($x10^4$)

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0

preimmune

anti-Bsp

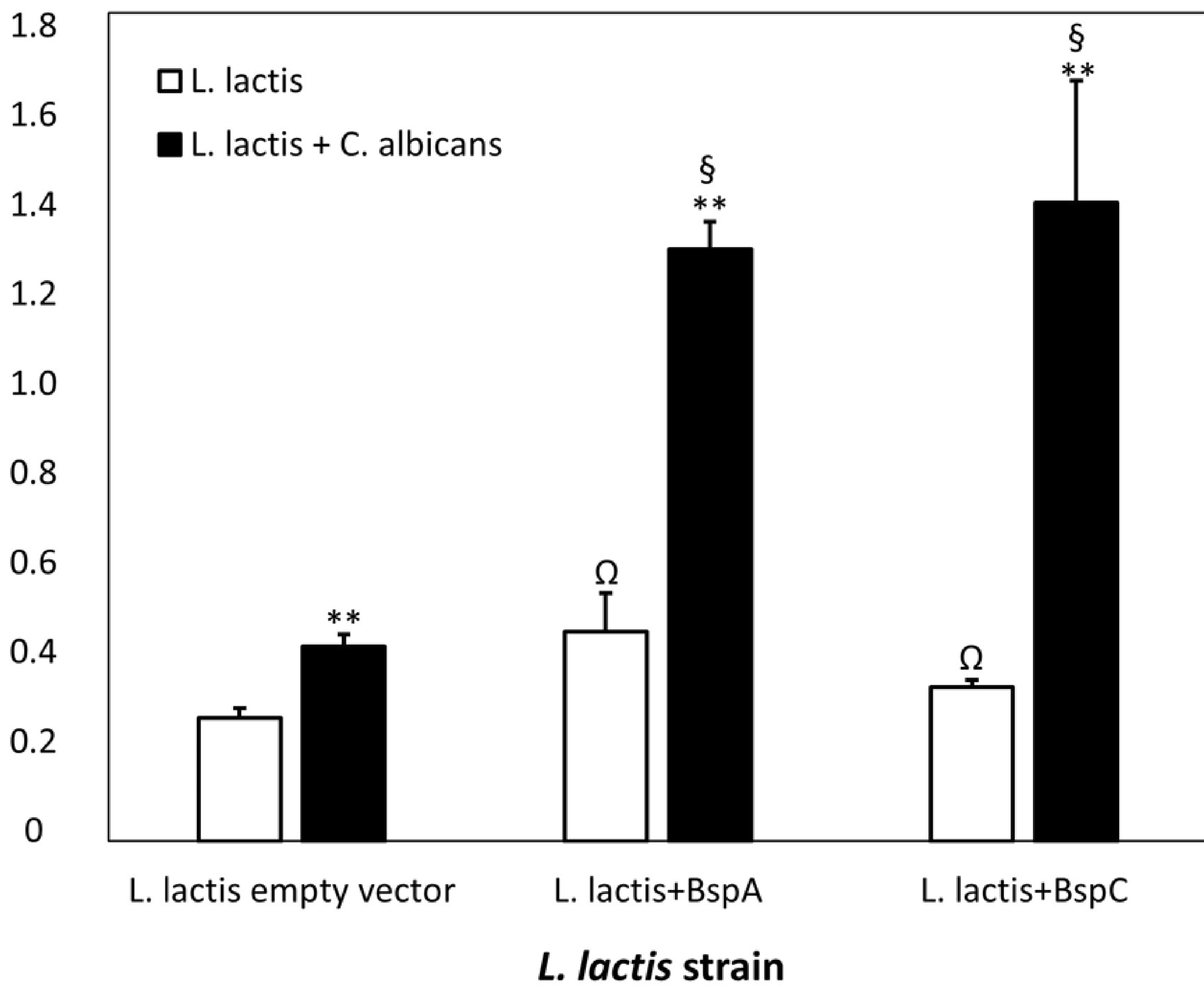
**

**

NEM316

515

GBS strain

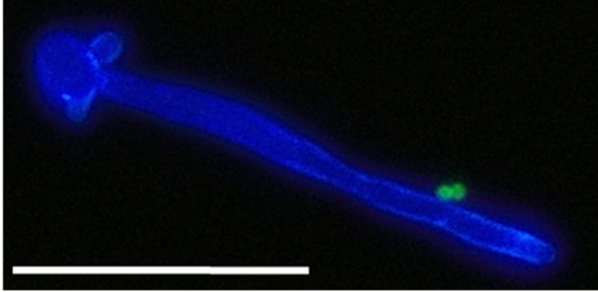


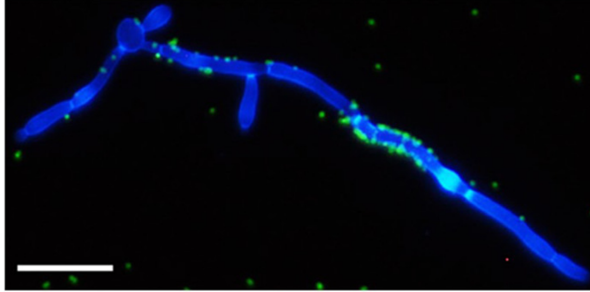
L. lactis
L. lactis + C. albicans
Number of associated L. lactis (x10^5)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
§
**
Ω
L. lactis empty vector
L. lactis+BspA
L. lactis+BspC
L. lactis strain

A

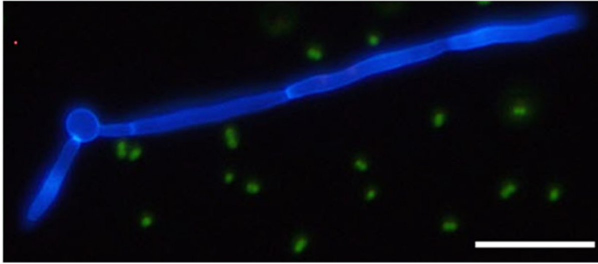
*C. albicans*Δ*als3* | *C. albicans*Δ*als3*+*als3*

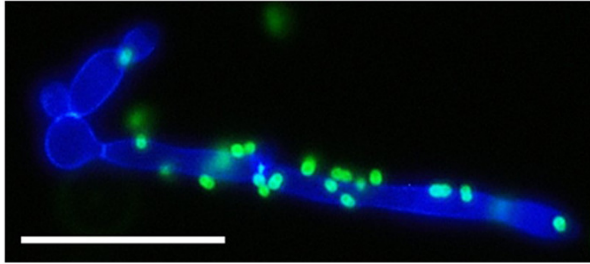
NEM316





515





B

% of interactions at each score

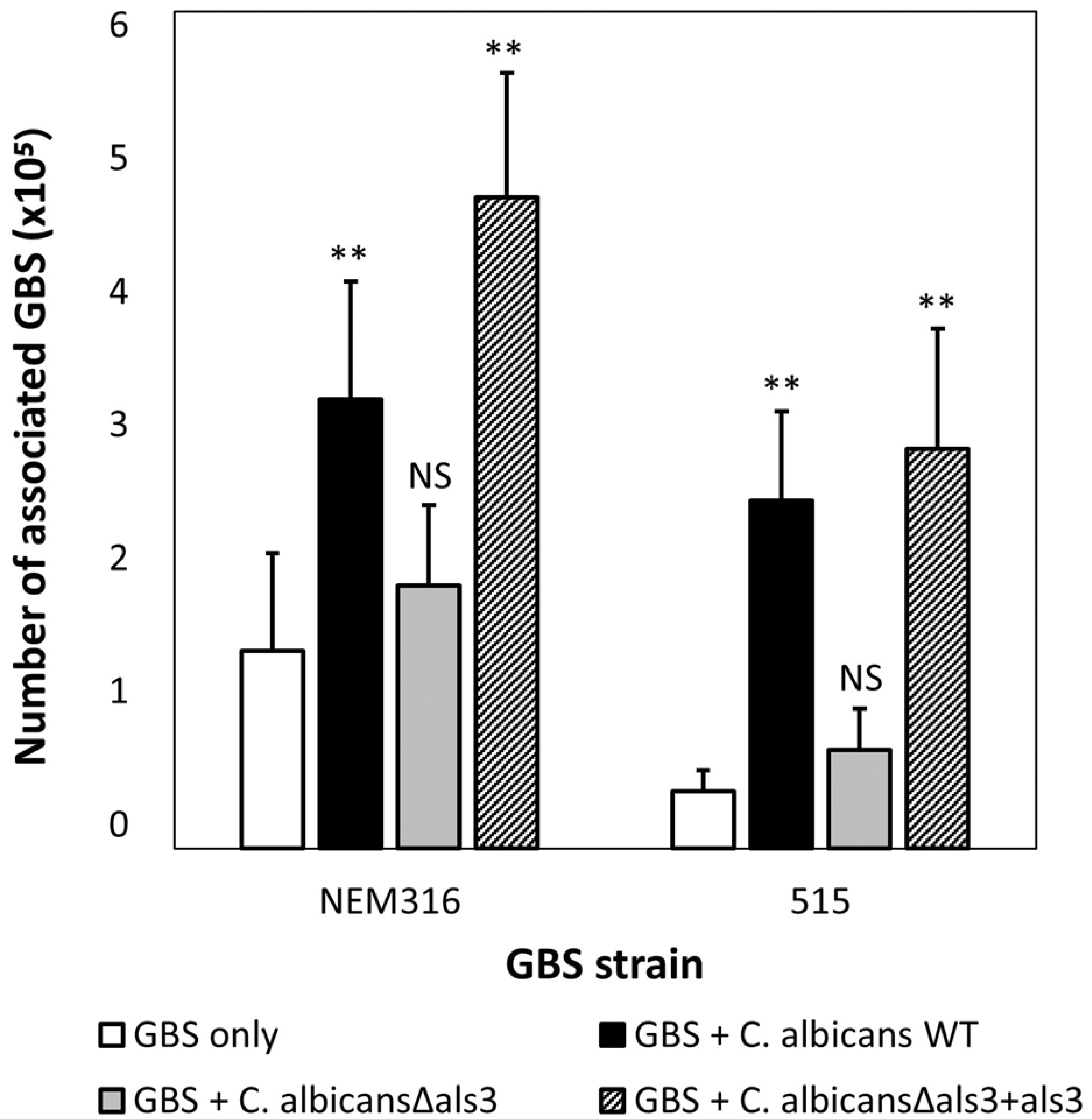
60
50
40
30
20
10
0

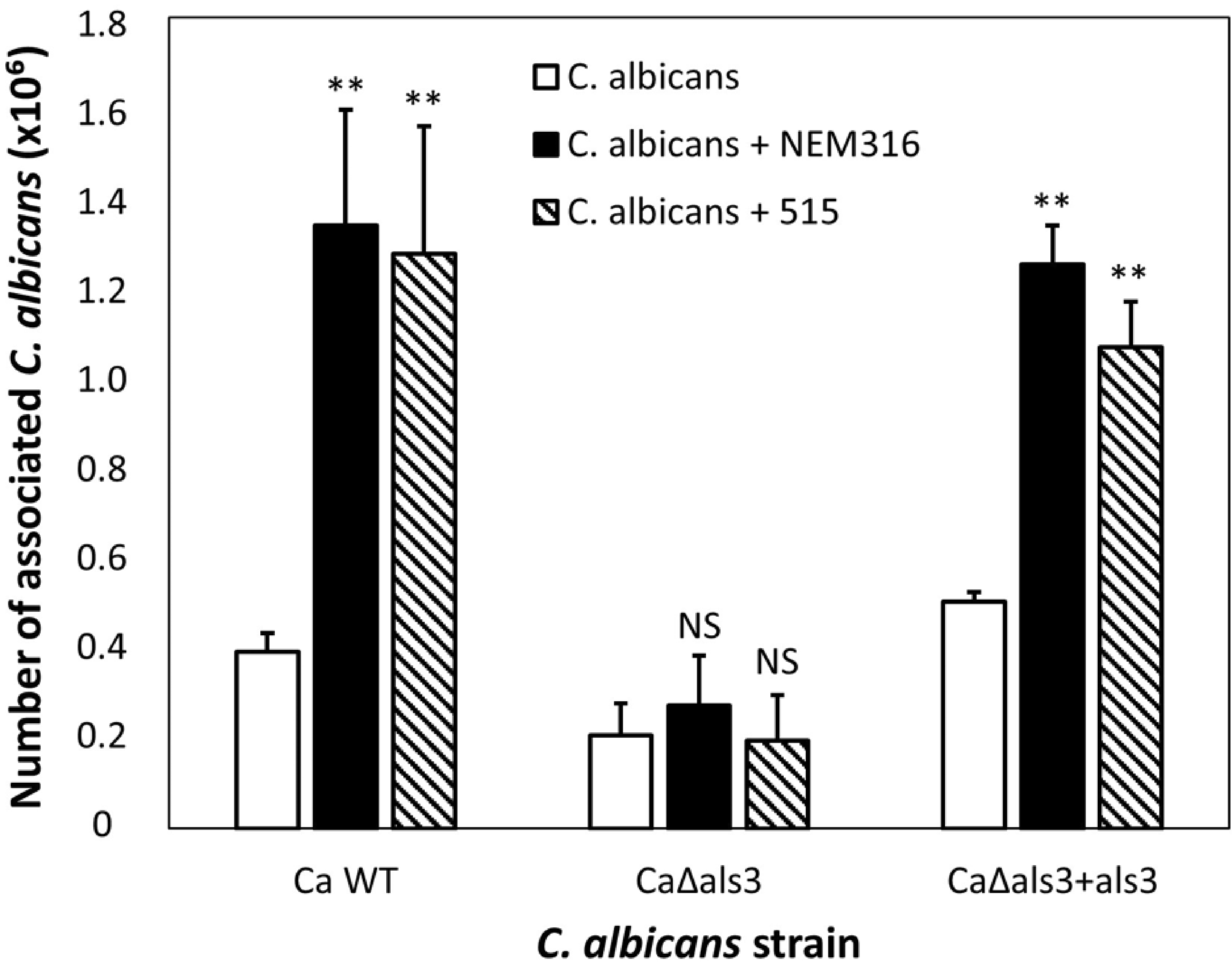
NS
*
NS
*

WT | Δals3 | Δals3+als3 | WT | Δals3 | Δals3+als3

NEM316 | 515

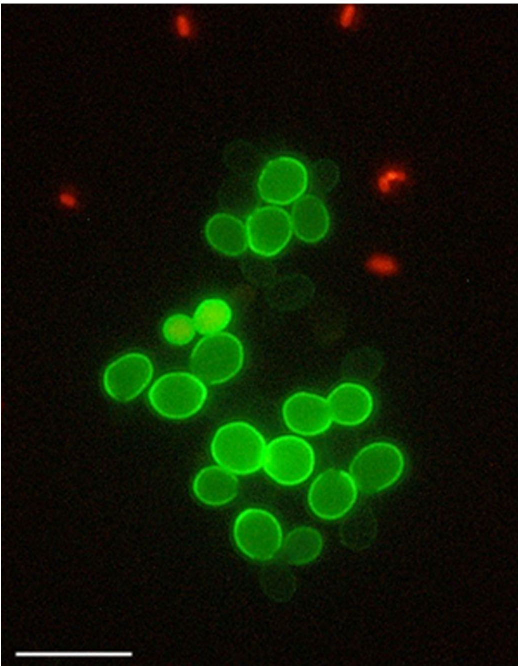
□ 0 interactions
▒ 1-5 interactions
▨ 6-20 interactions
■ >20 interactions


Number of associated GBS (x10⁵)
6
5
4
3
2
1
0
**
**
NS
**
**
NS
NEM316
515
GBS strain
GBS only
GBS + C. albicans WT
GBS + C. albicansΔals3
GBS + C. albicansΔals3+als3

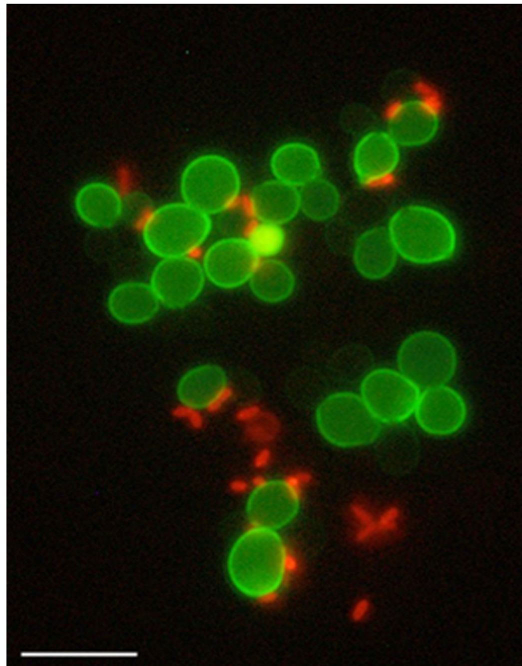


Number of associated C. albicans (x10⁶)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
C. albicans
C. albicans + NEM316
C. albicans + 515
**
**
**
**
NS
NS
Ca WT
CaΔals3
CaΔals3+als3
C. albicans strain

A) pMSP ctl



B) pMSP-*bspA*



C) pMSP-*bspC*