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Development of the Probiotic Yeast *Saccharomyces boulardii* as an Oral Vaccine Delivery System

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By

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Abstract

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By Lauren E. Hudson

Beneficial microorganisms can prevent and ameliorate the severity of numerous gastrointestinal disorders. Potential mechanisms of action of these so-called probiotics may include effects on the composition of the microbiota, reinforcement of the gastrointestinal epithelial barrier, modulation of mucosal immune responses, and direct anti-pathogen effects. Numerous microorganisms are currently studied and consumed typically as individual strains, but the ability to rationally design combination microorganism therapies tailored to particular diseases holds promise for further optimization of this adjuvant therapy. Recent studies also suggest the potential to use particular probiotics to synthesize and deliver oral vaccines and therapeutics. The yeast *Saccharomyces boulardii* may be particularly well suited to this purpose given its current consumption as a beneficial microbe, its ability to perform eukaryotic post translational modifications, and its lack of gastrointestinal colonization. In order to develop *S. boulardii* for this novel application of probiotic organisms, it will first be necessary to understand its interactions not with the inflamed or infected intestine, but with the healthy gut. The extent of *S. boulardii* uptake and interaction with mucosal immune cells may differ greatly in the healthy versus the inflamed intestine, potentially impacting responses to recombinant vaccine antigens. Furthermore, although genetic manipulation techniques for the closely related *S. cerevisiae* are well characterized, *S. boulardii* strains that can be manipulated without antibiotic selection and that can successfully express heterologous protein must be developed in order to serve as safe and efficient vaccine delivery vectors. Here we evaluate the interactions of *S. boulardii* with the healthy adult mouse intestine to provide insight into how this probiotic yeast may function as a vaccine delivery vector or prophylactic agent. We also present the generation of an auxotrophic mutant strain of *S. boulardii* that can be easily genetically manipulated without antibiotic selection markers and that can express heterologous protein. *In vivo* experiments also test the ability of recombinant auxotrophic mutant *S. boulardii* to induce immune responses specific to model vaccine antigens. These experiments thus provide a basis for further development and testing of *S. boulardii* as a vaccine delivery system to the mouse gastrointestinal tract.

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4) Introduction

The following is in part adapted from the article by Lauren E. Hudson, Sarah E. Anderson, Anita H. Corbett, and Tracey J. Lamb entitled "Gleaning Insights from Fecal Microbiota Transplantation and Probiotic Studies for the Rational Design of Combination Microbial Therapies" submitted to *Clinical Microbiology Reviews.*

Sections on *C. difficile* pathophysiology, colonization resistance, and fecal microbiota transplant were written by Sarah E. Anderson.

This dissertation discusses novel applications of probiotic organisms in two parts. The first part of chapter four presents a discussion of the need for rationally designed combinations of probiotic organisms as therapeutics for a range of gastrointestinal disorders. The second part of the introductory chapter and the following data chapters present the development of a probiotic strain of yeast, *Saccharomyces boulardii*, as a novel oral vaccine deliver vector. This dissertation thus describes novel applications of both wild type and adapted probiotics to treat and prevent gastrointestinal diseases.

a) Therapeutic Uses of Probiotics

Fecal microbiota transplant (FMT) is an effective and promising therapy for a number of gastrointestinal (GI) diseases, including *Clostridium difficile* infection (CDI) and inflammatory bowel disease (IBD). The simultaneous administration of a community of microorganisms in FMT is thought to exert therapeutic effects by restoring functions to the diseased intestine normally conferred by the native microbiota. The particular beneficial strains within FMT are currently incompletely defined, but an improved understanding of the therapeutic benefits conferred by individual microbial strains could enable tailored applications of microbial therapy that circumvent the logistical and ethical issues currently surrounding FMT.

Techniques of FMT administration vary, with fecal preparations given via nasogastric tubes, nasoduodenal tubes, colonoscopy, or enema [1]. Both related and unrelated donors have been used. Donor screening in either case is necessary to reduce the risk of spread of infectious diseases or other health conditions. Studies originally focused on using fresh feces, but frozen fecal preparations have also been shown to be effective [2] and this finding has facilitated the set up of stool banks as a source of preparations [3].

A recent review of case-series studies found no serious adverse events attributable to FMT [4], but this procedure is not entirely without risks. There have been two reported cases of patients contracting norovirus following FMT, although transmission was not linked to the donor in these cases [5]. There is also concern that FMT in immunocompromised patients could lead to acquisition of opportunistic infections, but the available data suggest that this is not a common problem for this patient population

115 [6,7]. However, there is evidence that FMT can lead to development of non-infectious
116 diseases. FMT for CDI has been linked to relapses in IBD [6,8] and to the development of
117 peripheral neuropathy, Sjogren's disease, idiopathic thrombocytopenic purpura, and
118 rheumatoid arthritis [9]. There has also been a reported case of development of obesity
119 following FMT from an overweight donor [10]. While donor screening is necessary to
120 reduce the risk of disease spread, recruitment and screening of donors is a difficult
121 process with low rates of success [11]. Development of treatments containing only the
122 effective components of FMT would alleviate many of these drawbacks that result largely
123 from the undefined nature of fecal preparations.

124 The focus of this review is to highlight the mechanisms of action by which
125 specific strains of microorganisms exert beneficial effects on the intestinal environment.
126 This information could be used to refine FMT into rationally designed combination
127 microbial therapies that will provide the benefits of FMT without the potential risks
128 associated with unknown components. Defined as live microorganisms that confer health
129 benefits when consumed, probiotic bacteria and yeast have been shown to reduce the
130 severity of several infectious and inflammatory diseases of the GI tract [12–15]. There are
131 several suggested mechanisms by which this protection may occur, including effects on
132 the composition of the resident microbiota, the GI epithelial barrier, and host immune
133 responses. However, in the context of particular diseases, certain functions may confer a
134 greater degree of benefit. Infectious diseases, for example, may require reinforcement of
135 the GI barrier, maintenance or restoration of a normal microbiota, and perhaps direct anti-
136 pathogen effects. In contrast, diseases with an autoimmune component may be mitigated
137 by probiotic strains that decrease inflammatory responses of the mucosal immune system.

138 Given that the potency of each of these potential mechanisms differs on a strain-specific
139 level, informed selection of probiotic strains to be administered therapeutically in place
140 of FMT is essential.

141 In this review, we use CDI and the inflammatory bowel disease ulcerative colitis
142 (UC) as illustrative cases to explore how microbial therapy might be tailored to either
143 infectious or autoimmune diseases. Both CDI and UC are serious GI diseases that are
144 increasing in prevalence [16,17]. Numerous trials have demonstrated effectiveness of FMT
145 for CDI, especially for recurrent infections, and recent smaller scale trials have suggested
146 that UC may also be treated with microbial therapy [16–18]. In the case of CDI,
147 pseudomembranous colitis arises from colonization with pathogenic *C. difficile* and direct
148 toxin-mediated damage of the host GI epithelium (Fig 4.1A and B). In contrast, UC
149 develops when genetically susceptible individuals exhibit breakdown of the GI barrier
150 due to aberrant inflammatory immune responses to microbial antigens [16] (Fig 4.1A and
151 C). Comparison of these two diseases with disparate pathogenic mechanisms allows for
152 consideration of how particular probiotic strains may be more appropriate in certain
153 disease contexts. We may thus gain insight into which particular organisms may best be
154 applied to the treatment of these and other infectious and inflammatory GI diseases.

155 To permit discussion of potential microbial therapeutics for infectious diseases as
156 exemplified by CDI and for inflammatory diseases as exemplified by UC, this review is
157 divided into two major sections. We begin each section with an overview of disease
158 pathophysiology followed by a discussion of applicable therapeutic traits identified for
159 particular probiotic and commensal organisms. Emphasis is placed on probiotic strains
160 for which clinical trials have been conducted for the diseases of interest, although

161 additional commensal strains shown to have potential benefits in experimental systems
162 are also considered. By identifying specific organisms with particular mechanisms of
163 action, we can inform studies and trials of rationally combined microbial therapeutics
164 tailored to individual infectious or inflammatory GI diseases.

165

166 **b) *C. difficile* Infection**

167 CDI is an increasing health problem, leading to nearly 500,000 diagnoses and
168 approximately 30,000 deaths annually in the United States alone [19]. *C. difficile* is an
169 obligate anaerobe but can survive for months in the external environment as dormant
170 spores [20,21]. Spores are highly resistant to many environmental stresses including ethanol-
171 based disinfectants [22]. In susceptible hosts, ingested spores germinate in response to bile
172 salts and amino acids found in the intestine [23]. Some individuals develop asymptomatic
173 colonization with *C. difficile*, while others develop pathogenic CDI. Symptoms of CDI
174 range from mild diarrhea to severe pseudomembranous colitis and death [24]. Both
175 asymptomatic and diseased individuals shed infectious spores in their feces that can then
176 spread and infect new hosts [25].

177 CDI is a toxin-mediated disease, and it has been suggested that patients
178 asymptomatically colonized by *C. difficile* may have more robust neutralizing immune
179 responses against *C. difficile* toxins than patients who develop symptoms [26]. Although
180 most *C. difficile* strains encode two toxins, TcdA and TcdB, strains have also been
181 isolated that produce only TcdB or no toxins; only strains without toxins are considered
182 to be avirulent [27–30]. TcdA and TcdB bind to any of a number of host cell receptors [31–34]
183 and, once inside host cells, act as monoglucosyltransferases to inactivate Rho family

184 GTPases [35,36]. This inactivation leads to rounding and death of GI epithelial cells,
185 disrupting the epithelial barrier [27,37,38]. Furthermore, although both toxins share these
186 similar targets and affect Rho GTPAses, they also have several differential effects on host
187 cells. TcdB, for example, induces necrosis of epithelial cells in an NADPH-dependent
188 mechanism [39]. TcdA impairs epithelial cell proliferation and repair by inhibiting the
189 Wnt/β-catenin pathway [40]. Some strains of *C. difficile* also encode a binary toxin, *C.*
190 *difficile* transferase (CDT) [41], which ADP-ribosylates actin and leads to actin
191 depolymerization and rearrangement of microtubules [41,42].
192 In addition to effects on cell death and proliferation, *C. difficile* toxins perturb the
193 intestinal epithelial barrier by affecting cytoskeletal components and junctional
194 complexes [43]. Both TcdA and TcdB mediate dissociation of the proteins zonula occludins
195 (ZO)-1 and ZO-2 in epithelial tight junctions, leading to separation of F-actin [44] and
196 modulating integrity of the epithelial barrier [45]. Influx of luminal compounds across the
197 intestinal barrier exposes immune cells to bacterial components as well as numerous
198 inflammatory damage-associated molecular patterns (DAMPs) from necrotic epithelial
199 cells. TcdA also disrupts epithelial cell polarization, thus affecting distribution of Toll-
200 like receptors (TLRs) and the nature and magnitude of immune responses to DAMPS [45].
201 Maintaining integrity of the junctional complexes between epithelial cells and reinforcing
202 the integrity of the epithelial barrier may thus help to limit damage induced by *C. difficile*
203 toxins and host inflammatory responses (Fig 4.4).
204

205 **1) Risk factors for developing CDI**

206 A healthy and intact gut microbiota decreases susceptibility to CDI, a

207 phenomenon known as colonization resistance [46]. Indeed, antibiotic exposure is the
208 primary risk factor for the development of symptomatic CDI because this treatment
209 perturbs the gut microbiota and reduces colonization resistance. Broad-spectrum
210 antibiotics are of greatest concern for the development of CDI; clindamycin,
211 cephalosporins, aminopenicillins, and fluoroquinolones are all particularly associated
212 with increased risk of CDI [47–49]. Antibiotic treatment depletes members of the two
213 dominant bacterial phyla in the gut, the Bacteroidetes and Firmicutes [50,51] . Antibiotics
214 also lead to increases in the numbers of Proteobacteria, which are associated with
215 susceptibility to CDI in humans [50–53]. Studies in both humans and animals have indicated
216 that changes in the microbiota brought on by antibiotic treatment can be long-lasting,
217 although this depends on the antibiotic used [50,51,54,55]. These changes in the gut microbiota
218 facilitate development of CDI following antibiotic therapy.

219 In addition to antibiotic use, other factors influencing susceptibility to CDI
220 include age, exposure to healthcare environments, and the use of proton pump inhibitors
221 such as for treatment of peptic ulcers. Asymptomatic colonization with *C. difficile* is
222 common in infants; in fact, it is estimated that up to 21-48% of infants are
223 asymptomatically colonized with *C. difficile* [56]. Asymptomatic colonization can also
224 occur in adults [56], but old age is a risk factor for development of symptomatic CDI [57–59].
225 The elderly are thought to be more susceptible because of changes in their gut microflora,
226 immunosenescence, increased exposure to healthcare environments, antibiotic use, and
227 other comorbidities [57,60]. Hospitalization is a major risk factor for both asymptomatic
228 carriage of *C. difficile* and acquisition of pathogenic CDI [20]. Proton pump inhibitors are

229 also thought to increase the risk of CDI by altering the composition of the gut microbiota
230 [61].

231 Natural anti-*C. difficile* TcdA and TcdB antibodies in the general population have
232 been proposed as a protective factor against disease development [62,63]. Toxin-reactive IgG
233 and IgA can be detected in the intestine and serum and have the potential to block toxin
234 binding to epithelial receptors and promote toxin clearance from the intestine [64]. The
235 presence of antibodies reactive to *C. difficile* TcdA has been positively correlated with
236 asymptomatic carriage of *C. difficile* [26]. In two reports, patients with recurrent CDI were
237 noted to have low levels of anti-toxin antibodies [65,66], supporting the hypothesis that
238 antibodies may confer some protection against disease. However, early studies noted both
239 healthy control and CDI patients to be positive for anti-*C. difficile* toxin antibody [62], and
240 subsequent studies presented conflicting results as to whether anti-toxin antibodies had
241 any effect on the course of the disease [26,63,68–70]. Still, a few studies showed successful
242 treatment of human CDI patients using intravenous human immunoglobulin therapy
243 (IVIG) [65,71–75], suggesting that antibodies can be beneficial. A retrospective review of 14
244 CDI patients found that 64% responded to IVIG therapy [76], although another study found
245 no difference in IVIG-treated patients versus those treated with standard antibiotic
246 regimens [77]. Questions thus remain as to the extent to which antibody levels may confer
247 protection against CDI.

248

249 **2) Treatment of CDI and Disease Recurrence**

250 Treatment for CDI generally involves withdrawal of offending antibiotics and
251 prescription of either metronidazole or vancomycin. Metronidazole is the preferred

252 treatment in mild disease due to its cost effectiveness, but it is associated with higher
253 rates of treatment failure relative to vancomycin in severe and complicated cases of CDI
254 [78]. A more recently developed antibiotic, fidaxomicin, has a similar cure rate as
255 vancomycin [79] but is currently recommended only for recurrent CDI due to its expense [78].
256 In particularly complicated cases, surgical intervention may be required to remove the
257 infected colon [78].

258 Recurrence of CDI following completion of treatment is common. This can occur
259 via a relapse of the initial infection or from re-infection with spores from the environment
260 [80,81]. Risk of recurrence is high because current therapies are limited to antibiotics that
261 kill much of the gut microbiota along with *C. difficile*. This wholesale killing results in
262 decreased colonization resistance due to suppression of levels of Bacteroidetes and
263 Firmicutes [82–84]. Vancomycin in particular has dramatic and long-lasting effects on the
264 composition of the microbiota [55]. Fidaxomicin, in contrast, has the least profound effect
265 on the gut microbiota [83] and is associated with lower rates of CDI recurrence than
266 vancomycin [79,83]. The serious problem of recurrence has led to interest in non-antibiotic
267 therapies to treat CDI, including microbial-based therapies such as FMT.
268

269 **3) Fecal Microbiota Transplantation and CDI**

270 FMT seeks to reconstitute a healthy gut microbiota and colonization resistance
271 against CDI through administration of fecal preparations from a healthy donor. A recent
272 review of case-series studies demonstrated that 85% of 480 patients with recurrent CDI
273 were successfully treated using FMT [4], illustrating the potential of using microbes as
274 therapy to restore colonization resistance against CDI. Still, the exact mechanisms of

275 colonization resistance and the commensal species conferring these benefits are not fully
276 understood. A few studies have successfully used defined bacterial consortia to cure CDI
277 in mice [85] and humans [86–88]; however, the role of each bacterial strain in restoring
278 colonization resistance was not examined. Understanding the mechanisms underlying the
279 efficacy of FMT for CDI could lead to development of more defined probiotic
280 therapeutics to reestablish colonization resistance and ameliorate disease.
281

282 **4) Clinical Trials Evaluating Probiotic Efficacy Against CDI**

283 Numerous clinical trials over the past few decades have evaluated the efficacy of
284 probiotics against *C. difficile* (Table 1), identifying some individual strains and cocktails
285 of beneficial microbes that may be candidates for further use in rationally designed
286 combined microbial therapies. These trials primarily tested lactic acid producing bacteria,
287 including *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species, and the probiotic
288 yeast *Saccharomyces boulardii*. Most studies have evaluated the ability of probiotics to
289 prevent primary CDI in patients receiving antibiotic therapy, although a few have
290 specifically considered prevention of recurrent CDI. The majority of trials to date have
291 been unable to determine a statistically significant benefit from probiotic administration
292 for the prevention of CDI. However, many studies are limited by a number of biases,
293 including lack of appropriate randomization, poorly defined outcome measures, and
294 reliance on *post hoc* analyses. Critically, most studies are small in scale and
295 underpowered [89]. Particularly in studies considering antibiotic-associated diarrhea as a
296 primary outcome and *C. difficile* infection as a secondary outcome, low incidence of CDI
297 in small study populations limits evidence of efficacy [90]. Results of individual trials are

298 also difficult to compare, as the selection and preparation of probiotic agents, treatment
299 length, study methods, patient populations, and means of identifying CDI cases all vary.
300 A recent large clinical trial tested use of *Lactobacillus acidophilus* (CUL60 and
301 CUL21) and *Bifidobacterium bifidum* (CUL20 and CUL34) in older patients receiving
302 antibiotic therapy and found no benefit in terms of diarrhea severity or abdominal
303 symptoms [91]. Nevertheless, a few earlier trials and meta-analyses found benefit of other
304 probiotic strains in treatment of CDI. One meta-analysis found a beneficial effect of using
305 *Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG, and certain probiotic mixtures
306 to reduce the risk of antibiotic-associated diarrhea and of using *S. boulardii* to reduce the
307 risk of CDI [12], although there has been some criticism of the trials included in this study
308 [92,93]. Of the four trials able to meet the stringent criteria of the Cochrane reviews in 2008
309 [94], only one showed a significant benefit of probiotics (*S. boulardii*) for preventing CDI
310 recurrence [95]. Thus, although transfer of the whole microbiota through FMT can be
311 effective in treating CDI, administration of currently available individual probiotic strains
312 and some cocktails do not appear to reliably confer protection.
313 Rational design of probiotic cocktails that provide the protective effects
314 associated with FMT while avoiding transfer of potentially deleterious strains would
315 provide a much needed therapy for CDI. Below we discuss individual strains associated
316 with colonization resistance and inhibition of the deleterious effects of *C. difficile.*
317 Further study of these strains could lead to development of effective probiotic therapies
318 for CDI.

319 **5) Microbial Taxa Associated with Colonization Resistance Against CDI**

320 Recent studies have attempted to identify individual commensal microbes
321 associated with colonization resistance or susceptibility to CDI in both humans and
322 animal models. This work has the potential to uncover taxa responsible for the efficacy of
323 FMT against CDI and that could be incorporated in future defined therapeutic cocktails.
324 Several studies have identified bacterial taxa associated with colonization resistance
325 versus the development of CDI in antibiotic-treated mice challenged with *C. difficile*. In
326 general, mice that remain healthy after challenge with *C. difficile* exhibit increased levels
327 of Firmicutes relative to mice that develop CDI [96]. The Porphyromonadaceae and
328 Lachnospiraceae families [99] and the genera *Lactobacillus, Alistipes*, and *Turicibacter* [97]
329 are also associated with colonization resistance against CDI in mice. In contrast, the
330 *Escherichia* and *Streptococcus* genera [97] and the Enterobacteriaceae family [96] correlate
331 with increased susceptibility to CDI . A recent analysis identified individual bacterial
332 species associated with colonization resistance in antibiotic-treated mice [98]. This study
333 identified *Clostridium scindens*, *Clostridium saccharolyticum*, *Moryella indoligenes,*
334 *Pseudoflavonifractor capillosus, Porphyromonas catoniae, Barnesiella intestihominis,*
335 *Clostridium populeti, Blautia hansenii,* and *Eubacterium eligens* as protective against
336 CDI [98]. The majority of these species belong to *Clostridia* cluster XIVa (phylum
337 Firmicutes) [98]. It has been suggested that members of *Clostridia* cluster XIVa may protect
338 against *C. difficile* colonization through their ability to metabolize bile salts, as discussed
339 below.

340 Human studies have also implicated particular microbial taxa in modulating
341 susceptibility to CDI. In general, high levels of the phylum Bacteroidetes, consisting of

342 strict Gram-negative anaerobes, are thought to be protective against CDI, whereas
343 increased numbers of Proteobacteria are thought to increase susceptibility [52,53,99,100].
344 These correlations are also consistent with observations that FMT recipients have
345 increased Bacteroidetes and decreased Proteobacteria following recovery from CDI [101].
346 More specifically, the family Ruminococcaceae and the genus *Blautia* are also associated
347 with colonization resistance to CDI, while multiple groups have found the family
348 Peptostreptococcaceae and the genera *Enterococcus* and *Lactobacillus* to be associated
349 with susceptibility [52,98,100,102].

350 Not all bacteria within the same group confer equivalent benefits to colonization
351 resistance in humans. Some taxa of the family Lachnospiraceae (belonging to Clostridia
352 cluster XIVa) are associated with protection in humans and mice [52,100,102], and FMT has
353 also been shown to increase levels of Lachnospiraceae [101,103]. However, some taxa within
354 this family are actually associated with increased susceptibility to CDI [100]. There are also
355 conflicting reports regarding the role of some bacteria. For example, some studies
356 associate *Streptococci* with colonization resistance [102] and others with susceptibility [52,98].
357 Such examples highlight the need for both experimental reproducibility and species- and
358 strain-level specificity when determining probiotic potential. In order to develop targeted
359 probiotics therapies, more studies will be needed to determine which bacterial strains are
360 able to confer colonization resistance.

361

362 **6) Mechanisms of Colonization Resistance Against CDI**

363 The mechanisms by which commensal bacteria mediate colonization resistance
364 against *C. difficile* are incompletely understood; however, several possible mechanisms

365 of colonization resistance are discussed below. It is likely that successful probiotic
366 therapeutics for CDI would restore colonization resistance by one or more of these
367 mechanisms.

368 ***1. Nutrient availability and competition for resources***

369 Commensal bacteria are thought to provide colonization resistance by occupying
370 nutrient niches that could be exploited by *C. difficile* [104]. Levels of nutrients and
371 metabolites in the mouse gut are substantially altered by antibiotic treatment [105,106],
372 presumably due to elimination of bacteria with specific metabolic functions. This change
373 in nutrient availability in turn favors *C. difficile* growth. Antibiotic treated, CDI-
374 susceptible mice exhibit elevated intestinal levels of carbohydrates [105] and sialic acid [107]
375 that enhance *C. difficile* growth. *C. difficile* has also been shown to consume succinate *in*
376 *vitro*, which is present at higher concentrations in mice following antibiotic treatment [108].
377 It is likely that restoration of the gut metabolome to a pre-antibiotic state through FMT is
378 a factor in restoring colonization resistance to CDI.

379 The concept of niche exclusion in the gut environment has led to interest in using
380 non-toxigenic *C. difficile* (NTCD) as a probiotic to prevent recurrent infections by
381 toxigenic strains. This strategy is based on observations that people asymptomatically
382 colonized with *C. difficile* are less likely than uncolonized individuals to develop
383 symptomatic CDI when hospitalized [109]. Administration of NTCD following clindamycin
384 treatment in the hamster model protects most animals from death due to challenge with
385 toxigenic *C. difficile* [110,111]. Human studies have also shown some promise for this
386 strategy: phase 1 clinical trials indicated that oral ingestion of the NTCD strain VP20621
387 is safe in healthy humans [112], and phase 2 trials showed that 11% of patients who

388 received VP20621 developed recurrent CDI relative to 30% of patients who received
389 placebo [113]. Although the mechanism by which NTCD is able to prevent colonization by
390 toxigenic *C. difficile* has not been thoroughly investigated, it is hypothesized that prior
391 NTCD colonization allows NTCD to outcompete newly-introduced toxigenic strains [113].
392 NTCD is thus an intriguing illustration of how certain bacterial species may occupy
393 particular niches within the gut and provide colonization resistance against toxigenic *C.*
394 *difficile*. However, it should be noted that the toxigenic strain 630Δ*erm* can share toxin
395 genes with NTCD strains via horizontal gene transfer *in vitro* [114]. Whether this transfer
396 would be a potential danger *in vivo* by converting NTCD to a toxigenic form remains to
397 be seen.
398
399 ***2. Bile salt metabolism and colonization resistance***
400 Levels of different bile salts in the gut are thought to affect *C. difficile*
401 colonization by directly modulating its germination and growth. The primary bile salts
402 glycocholate (GCA), glycochenodeoxycholate, taurocholate (TA), and
403 taurochenodeoxycholate are synthesized by the liver to aid in the breakdown, digestion,
404 and absorption of lipids in the small intestine (Fig 4.2) [115]. Although most bile salts are
405 reabsorbed in the ileum and recycled by the liver, about 5% of bile salts pass into the
406 large intestine, where they act as substrates for bacterial modification [115]. Primary bile
407 salts are deconjugated from their amino acid groups by bacterial bile salt hydrolases to
408 make cholate (CA) and chenodeoxycholate (CDCA) [115]. These can be further modified
409 by bacterial 7-hydroxysteroid dehydrogenases to form the secondary bile salts
410 deoxycholate (DCA) and lithocholate (LCA) [115]. Although a wide variety of bacteria are

411 capable of carrying out bile salt deconjugation, only a few intestinal bacteria can
412 synthesize secondary bile salts [115]. CDCA inhibits germination of *C. difficile*, while TA,
413 GCA, and CA all enhance *C. difficile* germination [23,116]. DCA is also capable of
414 enhancing germination of *C. difficile* spores, but inhibits the growth of vegetative cells
415 [23,116]. These findings suggest that levels of different bile salts in the intestine exert fine
416 control over *C. difficile* germination and outgrowth.

417 Antibiotic treatment results in bile salt level alterations that favor germination and
418 growth of *C. difficile.* Intestinal extracts from antibiotic-treated mice contain higher
419 levels of primary bile salts than extracts from untreated mice [105,117,118]. Spores incubated
420 with intestinal extracts from antibiotic-treated mice also germinate better than spores
421 incubated with untreated mouse extracts [117]. Addition of the bile salt chelator
422 cholestyramine to extracts eliminated *C. difficile* germination, showing that germination
423 occurs in response to bile salts in the mouse intestine [117]. Furthermore, patients with CDI
424 have higher levels of primary bile salts and lower levels of secondary bile salts than
425 healthy controls [119], and FMT has been shown to restore bile salt levels to those observed
426 in healthy individuals [120]. FMT efficacy thus appears to be mediated at least in part by
427 restoring normal bile salt metabolism in CDI patients.

428 The role of secondary bile acids in protecting against *C. difficile* colonization
429 suggests that bacteria with 7-hydroxysteroid dehydrogenase activity could be used as
430 probiotics against CDI. *C. scindens*, a Clostridia cluster XIVa bacterium that produces a
431 7α-hydroxysteroid dehydrogenase, has been associated with colonization resistance
432 against CDI in both mice and humans [98]. Administration of *C. scindens* to antibiotic-
433 treated mice restored DCA and LCA concentrations to pre-antibiotic levels, and intestinal

434 contents from these mice were shown to inhibit growth of vegetative *C. difficile* [98].
435 Furthermore, feeding antibiotic-treated mice *C. scindens* prior to challenge with *C.*
436 *difficile* significantly improved survival [98]. *C. scindens* may thus be an attractive
437 candidate for inclusion in novel probiotic formulations.
438
439 ***3. Production of anti-C. difficile compounds***
440 Production of molecules by the gut microbiota that have direct anti-bacterial
441 activity may also contribute to colonization resistance against *C. difficile*. Organic acids
442 produced by bacteria have been proposed to inhibit *in vitro* growth of *C. difficile*, with
443 culture supernatants from strains of *Lactobacillus*, *Lactococcus*, and *Bifidobacterium*
444 species demonstrating pH-dependent anti-*C. difficile* activity [121–123]. Growth of *C.*
445 *difficile* is also inhibited by supernatants from *Bacillus amyloliquefaciens* cultures [124].
446 Although the exact inhibitory molecule(s) within these culture supernatants remain to be
447 identified, antibiotic-treated mice given *B. amyloliquefaciens* prior to challenge with *C.*
448 *difficile* exhibit decreased disease [124], suggesting that these molecules are also active *in*
449 *vivo.* Lacticin 3147, produced by *Lactococcus lactis* strain DP3147 [125,126], and thuricin
450 CD, produced by *Bacillus thuringiensis* DPC 6431 [127], are both bacteriocins inhibitory to
451 *C. difficile*. Thuricin CD has potent activity against *C. difficile* without any apparent
452 significant effects on other gut commensals [84,127]; however, mouse studies suggest that *B.*
453 *thuringiensis* DPC 6431 spores pass through the mouse GI tract without germinating,
454 limiting the probiotic potential of this strain [128]. In contrast, lacticin 3147 is inhibitory
455 towards other gut commensals [125], likely limiting its potential for restoring colonization
456 resistance. More research is thus needed to identify strains that can both produce

457 compounds specific for *C. difficile* and remain in the *C. difficile*-infected GI tract long
458 enough to exert an effect.
459

460 **7) Other Mechanisms of Action of Beneficial Microbes and Probiotics**
461 **Against CDI**

462 ***1. Inactivation of C. difficile toxins***

463 Factors that directly target *C. difficile* toxins also have the potential to ameliorate
464 disease by limiting the damaging effects of CDI on the GI epithelium. *S. boulardii* has
465 been shown to secrete a 54 kDa protease capable of degrading TcdA, TcdB, and their
466 brush border membrane receptors *in vitro* [129,130]. Blocking this protease abrogated
467 protective effects of *S. boulardii* against *C. difficile* toxin-mediated epithelial damage *in*
468 *vitro* [130], suggesting that *S. boulardii* protects against CDI pathogenesis at least in part via
469 toxin degradation. However, this stands in contrast to another study that found no
470 survival increase in mice administered toxins preincubated with *S. boulardii* [131]. The role
471 of *S. boulardii* in direct toxin inactivation thus remains incompletely understood.

472 Probiotics also have the potential to inactivate *C. difficile* toxins indirectly by
473 increasing production of anti-toxin neutralizing antibodies. TcdA-reactive IgM and IgA
474 antibodies are induced by administration of *S. boulardii in vivo* [132]. One hypothesis is that
475 such antibodies could prevent TcdA binding to its receptors on epithelial cells, thus
476 limiting histological damage. This hypothesis is supported by a study in which a cocktail
477 of monoclonal antibodies directed against TcdA and TcdB was administered to hamsters
478 prior to *C. difficile* challenge. This approach was found to protect against GI damage and
479 death from CDI [133,134]. It is unclear whether organisms other than *S. boulardii* can also

480 induce antibodies with neutralization activity against *C. difficile* toxins. More studies are
481 needed to determine the degree of protection conferred by *C. difficile* toxin-specific
482 antibodies and to identify probiotic strains capable of stimulating such responses.
483
484 ***2. Antibody-mediated control of C. difficile bacteria***
485 Several studies have shown that administration of probiotic organisms can
486 increase total secretory IgA levels in rodents [132,135–137], which may contribute to control of
487 *C. difficile* bacteria[65,71–75]. *S. boulardii*, for example, increases total secretory IgA in
488 conventional rats and mice as well as in germ free mice colonized with *S. boulardii*
489 [132,135–138]. Studies of *Bifidobacterium animalis var. lactis* BB-12, *Escherichia coli* EMO,
490 and *Lactobacillus casei* and *L. rhamnosus* strains showed effects on total secretory IgA
491 levels in rodent models [136,139–141]. However, more studies are needed to determine
492 whether these changes in antibody production could prove protective against CDI.
493
494 ***3. Inhibition of mucus layer disruption***
495 Mucus forms a semipermeable barrier between the GI epithelium and the lumen.
496 It consists of mucin glycoproteins, which are produced by goblet cells within the
497 epithelium [142]. The secreted glycoprotein, MUC2, and membrane-bound mucins MUC1,
498 MUC3, and MUC17 form a dense meshwork into which numerous bioactive molecules,
499 including trefoil factor peptides, resistin-like molecule β (RELMβ), Fc-γ binding protein,
500 and antimicrobial peptides, as well as commensal bacteria are able to bind [143,144]. This
501 mucus barrier normally prevents direct contact of bacteria with the epithelium.

502 CDI is associated with changes in mucus thickness and composition [145] that
503 promote *C. difficile* binding to mucus and increase the risk of epithelial damage from *C.*
504 *difficile* toxins [146–149]. Intestinal biopsies from CDI patients show decreased MUC2
505 expression relative to healthy patients [145]. *C. difficile* and CDI stool samples decrease
506 MUC2 and alter mucus oligosaccharide composition in cultured human intestinal
507 epithelial cells [145]. Incubation with TcdA also decreases mucin exocytosis from the
508 HT29- Cl.16E human colonic goblet cell line [150]. As such, a key mechanism of FMT and
509 probiotics in protecting against CDI may be to restore mucus composition in order to
510 maintain an effective barrier.

511 A limited number of probiotics have been well studied with regards to modulation
512 of mucin production. Intestinal epithelial cells exposed to *Lactobacillus plantarum* 299v
513 or LGG have been shown to upregulate MUC2 [151] and MUC3 expression [152],
514 respectively. In the case of LGG, this upregulation is mediated via the secreted soluble
515 protein p40, which activates the epidermal growth factor receptor and induces mucin
516 expression from GI epithelial cells [153]. Preincubation of epithelial cells *in vitro* with *L.*
517 *rhamnosus* ATCC 7469 has been shown to maintain mucin expression upon incubation
518 with enterotoxigenic *E. coli* (ETEC) [154]. Interestingly, an increase in mucus layer
519 thickness via addition of exogenous mucus increased the ability of *L. rhamnosus* to
520 prevent adherence and pathogenic effects of ETEC, suggesting that an intact mucus layer
521 may support the protective effects of probiotics. Induction of increased mucus and mucin
522 expression have also been noted for the probiotic bacteria cocktail VSL#3 incubated with
523 HT-29 cells *in vitro* [155] as well as *in vivo* when fed to laboratory rats [156]. A probiotic yeast
524 strain, *S. cerevisiae* CNCM I-3856, also upregulates MUC1 mRNA expression in

525 epithelial cells *in vitro* [157], possibly via the induction of butyrate [158,159]. However, some
526 probiotic strains such as *E. coli* Nissle 1917 have minimal effects on mucus [155]. In
527 addition to species differences, the *in vivo* ability of particular probiotics to affect the
528 mucus layer may furthermore differ depending on the age [160] and overall GI microbiota
529 composition [161] of patients. Thus, currently only some probiotic strains are clearly
530 capable of influencing mucus production, and more research is needed to evaluate their
531 effects on restoring mucus specifically in the context of CDI.
532

533 ***4. Maintenance of the intestinal epithelial cell barrier and tight junction***
534 ***expression***

535 Microbes may promote maintenance of the epithelial barrier between luminal
536 contents and host cells through the modulation of mucus production (as discussed above)
537 or by influencing regulatory factors, such as cytokines, that affect intestinal permeability
538 (see discussion below on the cytokine milieu). However, many probiotics have also been
539 shown to influence the barrier function of epithelial cells by modulating expression of the
540 junctional complexes (Table 2). These complexes normally seal together adjacent
541 epithelial cells and prevent indiscriminate translocation of particles from the gut lumen
542 into host tissues. Although the role of junctional complexes in the pathogenesis of CDI is
543 not well studied, it is possible that reinforcing the GI epithelial barrier via modulation of
544 junctional complexes may help to reduce leakiness associated with CDI-induced
545 inflammation and possibly help repair damage induced by *C. difficile* toxins.

546 Junctional complexes are composed of tight junctions, adherens junctions, gap
547 junctions, and desmosomes [162] (Fig 4.3). Most work on junctional complexes and

548 probiotic organisms has centered on tight junctions, whose transmembrane components
549 include claudins, occludins, and junction associated molecule (JAM) family proteins.
550 These transmembrane components interact with plaque proteins, including zonula
551 occludens (ZO) family members [163], in order to mediate intracellular signaling and
552 cytoskeletal reorganization [164,165]. Expression of tight junction molecules in the healthy
553 gut is modulated by numerous environmental signals, including metabolic compounds
554 such as acetate and short chain fatty acids (SCFAs) [166], although the exact mechanisms
555 by which this occurs are still unclear. Some studies suggest that butyrate, an SCFA that
556 increases with probiotic administration, decreases intestinal permeability through
557 induction of AMP-activated protein kinase activity and increased assembly of tight
558 junctions in Caco-2 monolayers [167]. Junctional complex expression is also influenced by
559 innate immune functions of epithelial cells such as by TLR recognition of microbial
560 ligands [168].

561 Several probiotic organisms are capable of modulating junctional complexes to
562 restore or maintain the intestinal epithelial barrier. The probiotic yeast *S. boulardii*
563 increases expression of ZO-1 in T84 cells [169] and has been associated with decreased
564 intestinal permeability in numerous studies [138,170–173]. Similarly, *Bifidobacterium longum*
565 and LGG have both been shown to induce upregulation of claudin-1, ZO-1, and occludin
566 protein levels in keratinocytes [168]. Intriguingly, the *in vitro* increase in keratinocyte TER
567 induced by *B. longum* lysate, but not by *L. rhamnosus* GG lysate, was abrogated in the
568 presence of a TLR2-neutralizing antibody, suggesting that these bacteria act on different
569 pathways to influence tight junction molecule expression [168].

570 There is evidence that the effects of probiotics on epithelial cell junctional
571 complexes are highly strain specific. One study using Caco-2 cells exposed to probiotics
572 found that while all tested *Bifidobacterium* strains increased transepithelial electrical
573 resistance (TER), a measure of barrier integrity, only 6 of 15 tested *Lactobacillus* strains
574 showed a similar increase [174]. Even fewer strains were able to prevent the TNF-α-induced
575 decrease in TER [174]. Furthermore, the effect of the most protective strain of *B. bifidum*
576 (WU12) on TER was strikingly attenuated when heat-killed, suggesting that metabolic or
577 secreted factors produced by *B. bifidum* mediate beneficial effects[174]. Another study
578 found *L. plantarum* L2 was able to reduce TNF-α levels, intestinal epithelial apoptosis,
579 and ileal mucosal erosion in an ischemia reperfusion injury model [175]. By reinforcing the
580 GI epithelial barrier, probiotic organisms may help to repair or prevent damage induced
581 by *C. difficile* toxins or host inflammatory immune cells.
582

583 **8) Summary of Potential Mechanisms of Action of FMT Against CDI and**
584 **Implications for Probiotics**

585 *C. difficile* infection is a toxin-mediated disease that leads to severe damage of the
586 GI mucosa (Fig 4.4). Numerous factors may help to prevent initial colonization with *C.*
587 *difficile* or to maintain an asymptomatic infection and limit damage after sporulation in
588 susceptible individuals.

589 The phenomenon of colonization resistance in preventing CDI is particularly well
590 studied and presents one major mechanism through which beneficial microbes may help
591 to ameliorate disease pathogenesis and symptoms. Strains able to alter bile salt
592 concentrations or limit the availability of other resources may discourage growth and

593 colonization of *C. difficile*. Delivery of NTCD is also a promising novel therapy due to its
594 potential competition with toxigenic *C. difficile* for an intestinal niche [110,111]. Future work
595 administering *C. scindens* and NTCD [112,113] holds promise for use of these strains as
596 preventative therapies in antibiotic-treated patients or as treatments for CDI. Further
597 studies on colonization resistance will help identify additional microbes that could be
598 beneficial in treating CDI.

599 Direct targeting of *C. difficile* or its toxins is another way by which probiotics
600 may protect against CDI even after pathogen colonization and sporulation. Indeed, the
601 probiotic yeast *S. boulardii* has been found to secrete a protease capable of degrading *C.*
602 *difficile* toxin A [129,130]. It is interesting to note that this is the only probiotic strain for
603 which such direct anti-*C. difficile* toxin activity has been identified and one of the few
604 strains shown to have efficacy against CDI in clinical trials [95 94]. Identification of other
605 yeast or bacterial strains with anti-toxin activity may provide further potential therapeutic
606 strains.

607 Other probiotic strains may help to ameliorate disease symptoms and limit
608 damage by promoting reinforcement and repair of the epithelial barrier. Such
609 reinforcement may help protect the host from increased exposure to *C. difficile* toxins. *In*
610 *vitro* studies also suggest that effects of probiotics may be greater with an intact mucus
611 layer, suggesting that probiotics may be more beneficial as prophylactic agents. However,
612 further studies are needed to determine whether the effects of these probiotic strains seen
613 *in vitro* confer protection in the context of CDI.

614 Finally, administration of probiotic organisms may be beneficial by harnessing
615 the host immune response to alleviate CDI disease progression and symptoms. For

616 example, increasing production of secretory IgA may promote sequestration of toxins
617 within the intestinal lumen [65,71–75,132]. Stimulation of pattern recognition receptors (PRRs)
618 such as TLRs has also been found to limit CDI severity [176]. However, such a strategy
619 must be pursued with care: it has also been hypothesized that some degree of damage in
620 CDI may be immune-mediated, with decreased toxin-associated damage seen in mice
621 deficient in neutrophils, mast cells, or the inflammatory cytokine IFN-γ [176]. Probiotic
622 strains able to attenuate inflammatory responses may thus limit host-induced histological
623 damage and improve disease symptoms. In order to identify optimal probiotics for
624 treatment of CDI, it will be crucial to identify those strains able to alleviate symptoms
625 associated with deleterious inflammatory responses without undermining the ability to
626 control C. *difficile* infection. Current knowledge of immunomodulatory effects of
627 probiotics and implications for their use in GI diseases are discussed in further detail
628 below in the context of UC.
629

630 **c) Ulcerative colitis**

631 Ulcerative colitis is a serious GI disorder currently affecting an estimated 1-1.3
632 million people in the United States [177,178]. UC is more common in developed countries
633 and in urban areas. Incidence and prevalence of UC and Crohn's disease (CD), another
634 common form of IBD, are both highest in northern Europe and North America; however,
635 incidence is also increasing in other world regions, including South America and Africa
636 [177]. Although often grouped together with CD, UC is a distinct etiology from CD with
637 different associated genes, inciting factors, responses to therapies, and affected bowel
638 regions [16,179].

639 UC pathology is characterized by diffuse mucosal inflammation and histological
640 alterations limited to the mucosal layer of the colon [180]. The inflammation seen in UC is
641 chronic, but waxes and wanes in intensity. Varying degrees of immune cell infiltration
642 may be observed in the mucosa depending on whether the individual is experiencing
643 active disease or remission [16]. In active disease, lymphocytes, plasma cells, and
644 granulocytes may all be seen within the mucosa [181]. Ulcerations, goblet cell depletion,
645 and fewer crypts are also observed. In advanced disease, epithelial cells may undergo
646 dysplasia and increase risk of epithelial cancer [182,183]. Symptoms of mild to moderate
647 disease may include rectal bleeding, diarrhea, and abdominal cramping, while more
648 severe cases may present with fever, weight loss, anemia, and severe abdominal pain [16].
649 UC may also cause extra abdominal symptoms affecting the eyes, kidneys, and joints [184].
650

651 **1) Risk factors for developing UC**

652 Development of UC is thought to be a multi-hit process, with genetic
653 predispositions leading to disease only upon exposure to as yet poorly understood
654 environmental triggers. Several genetic correlations have been identified, with a recent
655 meta-analysis identifying 47 loci associated with IBD, 19 of which were specific for
656 susceptibility to UC rather than CD [179]. Still, twin studies have shown that overall genetic
657 concordance for UC is low relative to CD and other genetic diseases [16]. Environmental
658 exposures related to Western diet and lifestyle have also been linked to development of
659 UC [185,186]. Other known epidemiological risk factors include appendectomy [187]and
660 smoking [188], both of which reduce disease risk.

661

662 **2) Ulcerative Colitis Pathophysiology**

663 Although the exact mechanisms of UC pathogenesis are still incompletely
664 understood, disease is generally believed to stem from inflammatory immune responses
665 to the microbiota in genetically susceptible individuals [189]. The major contributing factors
666 to active disease are thought to include impaired barrier integrity of the GI epithelium, an
667 altered microbiota, and aberrant immune responses to GI antigens and microbes; these
668 factors are discussed in more detail below (Fig 4.5). Other factors that may also play a
669 role, such as adiposity, regulatory RNA, angiogenesis, and the inflammasome, have been
670 reviewed elsewhere [190] and are not discussed here.

671

672 ***1. Intestinal permeability***

673 Intestinal permeability is a major component of UC pathology and may serve as a
674 potential novel therapeutic target [191]. Breakdown of the epithelial barrier may lead to
675 increased and prolonged exposure to bacterial antigens or other insults that in turn may
676 compound inflammatory responses and intestinal damage. Whether intestinal
677 permeability is a cause or consequence of disease is still a question of debate. However,
678 several genome wide association studies (GWAS) have identified numerous UC
679 susceptibility loci that contain genes involved in intestinal permeability and pathogen
680 recognition, suggesting a causative effect [179,192,193]. Many of these genes are known to be
681 expressed by epithelial cells, including *GNA12*, which is associated with tight junction
682 assembly [179]; *CDH1*, encoding the adherens protein E-cadherin [179,192]; and *LAMB1*,
683 encoding the laminin beta 1 subunit expressed by the intestinal basement membrane.

684 Some studies have also found UC susceptibility to be associated with polymorphisms in
685 the multidrug resistance 1 gene (MDR1, also known as ABCB1) encoding P-
686 glycoprotein, a protein responsible for pumping substances out of epithelial cells to help
687 maintain barrier function [193,194].

688 The mucus layer that forms an additional barrier between epithelial cells and the
689 GI lumen is dysregulated and thinned in UC [195,196]. This is proposed to be the result of
690 defects in mucin production as well as increased numbers of mucus-degrading
691 (mucolytic) bacteria in individuals with UC [197]. Indeed, MUC2-deficient mice
692 spontaneously develop colitis, demonstrating the need for this factor in maintenance of
693 gut homeostasis [195]. The nod-like receptor pyrin domain-containing protein 6 (NLRP6),
694 which is known to be important in mucin exocytosis from epithelial cells, has also been
695 linked to colitis susceptibility in mouse models [198,199]. UC patients have significantly
696 reduced numbers of mucin-containing goblet cells in uninflamed ileal biopsies relative to
697 controls [200], suggesting dysregulation of mucus production occurs even in the absence of
698 host inflammatory cell responses. Decreased mucus layer thickness allows for increased
699 contact between the microbiota and the epithelium in UC patients [201], and may exacerbate
700 immunostimulation and inflammation.

701

702 ***2. The microbiota and dysbiosis***

703 The microbiota of UC patients is vastly different from those of healthy controls,
704 although it is unclear whether this is a cause or consequence of the chronic inflammation
705 associated with UC. Dysbiosis may be influenced by genetic risk factors leading to

706 impaired intestinal epithelial integrity as well as dietary factors such as high intake of fat,
707 refined sugar, iron, and aluminum [202].

708 There are alterations in several bacterial groups within the microbiota of UC
709 patients relative to healthy individuals. Like those suffering from CDI, UC patients have
710 decreased prevalence of Bacteroidetes and Firmicutes and increases in Actinobacteria and
711 Proteobacteria, especially Enterobacteriaceae [203,204]. UC patients were also specifically
712 found to have increased Porphyromonadaceae and enteroadherent *E. coli* in addition to
713 decreased *Prevotella*, *Catenibacterium*, *Streptococcus*, and *Asteroleplasma* species
714 relative to healthy patients [204,205]. Patients with active UC disease have also been reported
715 to have decreased prevalence of *Lactobacillus* species relative to patients in remission
716 [206].

717 The mechanisms by which dysbiosis influences the development of UC are
718 currently unclear; however, it is possible that dysbiosis early in life may predispose
719 individuals to UC by negatively affecting the maturation of the immune system [207]. GI
720 immune tissues such as Peyer's patches, isolated lymphoid follicles, and mesenteric
721 lymph nodes are all underdeveloped in the absence of microbial stimulation [208]. Indeed,
722 models known to develop spontaneous colitis, including interleukin (IL) -10 and T cell
723 receptor deficient mice, do not develop colitis if raised in germ-free conditions [208–210],
724 indicating that aberrant immune responses to a deregulated microbiota play a role in
725 inciting colitis. Furthermore, co-housing wild type mice with colitis-prone *Tbx21*$^{-/-}$ *Rag*$^{-}$
726 $^{/-}$ mice induces development of colitis in the wild type mice [208]. Although the exact
727 signaling pathways through which this susceptibility is conferred are still unclear, this
728 data suggests that exposure to certain colitogenic strains of bacteria within a dysbiotic

729 microbiota can be sufficient to induce colitis. Together these studies demonstrate that
730 dysbiosis is both a consequence of immune deregulation and a factor that affects disease
731 susceptibility and progression.

732

733 ***3. Aberrant immune responses***

734 UC is characterized by the infiltration and activation of many immune cells in the
735 mucosa, including neutrophils, macrophages [211], and T cells [212]. These inflammatory cells
736 are recruited and activated by the production of numerous chemokines and cytokines that
737 are upregulated in the mucosa of UC patients, further promoting inflammation and
738 damage in active disease [213]. Serum levels of chemokines that attract monocytes,
739 dendritic cells, T cells, and neutrophils, including CXCL5 and CCL23, are elevated in
740 UC patients compared to healthy controls [214]. Macrophage migration inhibitory factor
741 (MIF), macrophage inflammatory protein-3 (MIP3, CCL23), monocyte chemoattractant
742 protein-1 (MCP-1, CCL2), macrophage inflammatory protein-3 beta (MIP3b, CCL21),
743 and granulocyte chemotactic protein-2 (CXCL6) are also elevated in the periphery of UC
744 patients [214]. CCL25-CCR9 interactions, which regulate leukocyte recruitment to the
745 intestine, also play a role in mediating colitis [215]. Novel antibodies such as vedolizumab
746 and PF-00547659, which prevent homing of leukocytes to the gut, have been found to
747 ameliorate symptoms of active UC in clinical trials [16]. The ability to modulate immune
748 cell recruitment and the level of inflammatory cytokines may thus confer protection
749 against disease severity.

750

751 ***4. TNF-α***

752

753 In addition to their role in immune cell recruitment, inflammatory cytokines can
754 be directly pathogenic. The best example of this is TNF-α, which promotes fibroblast
755 proliferation, increased adhesion molecule expression, neutrophil activation, disruption of
756 junctional complexes, and production of pro-inflammatory cytokines such as IFN-γ [216].
757 UC patients have increased levels of TNF-α relative to healthy controls [217].
758 Administration of infliximab, a monoclonal antibody against TNF-α, has shown some
759 success in the treatment of steroid-refractory UC [218], highlighting the critical role of this
760 cytokine in mediating disease pathogenesis.

761

762 ***5. Th2 cells***

763 Despite the abundance of pro- and anti-inflammatory cytokines such as IL-12,
764 TNF-α, IL-1β, IL-16 and TGF-β that can be found in UC patients [214,217,219–221], UC has
765 traditionally been considered a $CD4^+$ T helper cell type 2 (Th2) disease [222,223]. This view
766 stemmed from the observation that increased levels of Th2-associated cytokines,
767 including IL-5 and IL-13, can be measured in UC patients and experimental colitis
768 models [219,223,224]. Th2-associated cytokines have been shown in some studies to induce
769 damaging effects at the mucosa. IL-13, for example, is thought in some situations to
770 mediate epithelial cell cytotoxicity, apoptosis, and barrier dysfunction [219,225]. However,
771 the importance of Th2 cytokines in UC pathogenesis relative to other cytokine pathways
772 is currently unclear.

773

774 ***6. Th17 cells***

775 Recent evidence also suggests an important role for Th17 cells, a subset of $CD4^+$
776 T cells that secrete primarily IL-17 [222,223], in UC pathogenesis. Th17 cells and their
777 associated cytokines increase neutrophil recruitment to areas of inflammation as
778 discussed below; however, the extent to which Th17-associated cytokines such as IL-17A
779 are pathogenic versus protective is controversial [226]. A recent GWAS identified numerous
780 Th17-related genes associated with UC susceptibility [179]. Multiple genes in the IL-23
781 pathway that induces Th17 cell differentiation, including *IL23R*, *JAK2*, *STAT3*, and
782 *IL12B,* were also associated with susceptibility to both UC and CD [179]. Both rodents with
783 colitis and patients with active UC disease have increased IL-17 and Th17 cells in the
784 mucosa relative to controls [227–229]. Although some studies have shown that antibody
785 depletion of IL-17 increases the severity of acute colitis in mice [230], other mouse
786 experiments conversely demonstrated that IL-17R deficiency reduces colitis severity [231].
787 Novel drugs blocking IL-17 activity have also been shown to confer protection in models
788 of chronic colitis [232,233]. Thus there is currently much evidence to suggest a critical role
789 for Th17 cells and their associated cytokines in UC pathogenesis.
790

791 ***7. Neutrophils***

792 The recruitment and activation of neutrophils at the intestinal mucosa is a striking
793 feature of UC pathophysiology [234,235]. Neutrophils are innate immune cells that normally
794 protect the host against microbial pathogens and dying cells through pathogen
795 phagocytosis and the production of reactive oxygen species, antimicrobial peptides, and
796 proteases such as elastase that are exuded from specialized granules. Numbers of

797 neutrophils are increased in both the periphery [236] and colons [237] of UC patients.
798 Neutrophils secrete both pro-inflammatory factors such as IL-17 [238], leukotrienes, and
799 CXCL8 [237] and also anti-inflammatory cytokines such as IL-10 [239]. Matrix
800 metalloproteases, which are involved in activation of chemokines such as CXCL5 and
801 CXCL8, are also secreted by neutrophils to facilitate the recruitment of additional
802 immune cells.

803 The exact role played by neutrophil expansion and activation in UC pathogenesis
804 has been the subject of much debate with different experimental colitis models suggesting
805 different effects of neutrophils on disease severity. Neutrophils are important in wound
806 healing and maintenance of homeostatic processes through their phagocytosis of
807 damaging cellular debris as well as through the secretion of growth promoting factors
808 such as vascular endothelial growth factor (VEGF), lipoxins, and protectins [237]. Some
809 studies have reported that depletion of Gr1+ CD11b+ cells, including neutrophils,
810 exacerbates mouse models of colitis, suggesting a protective role for neutrophils [240,241].
811 However, other studies have demonstrated the opposite effect [242] perhaps due to
812 differences in neutrophil depletion methods.

813 Although neutrophils are normally short lived cells, buildup of neutrophils in
814 chronic UC inflammation can overwhelm the ability of resident macrophages to clear this
815 cell population, leading to neutrophil necrosis and release of damaging granule contents
816 [237]. Thus, the ability of certain factors to either inhibit (IL-8, IL-1, IFN-γ, GM-CSF, and
817 C5a) [237,243] or promote (IL-10, TNF-α) [244,245] neutrophil apoptosis can influence the
818 degree of tissue damage. The massive transmigration of neutrophils through the
819 epithelium and release of elastase has also been associated with decreased expression of

820 tight junction and adherens junction proteins [246]. Elevated levels of fecal elastase have
821 been found to correlate with disease severity in UC patients [247]. It thus appears that
822 neutrophils may be contributors to both disease pathogenesis and recovery in UC.

823 **3) Treatment of UC**

824 Unfortunately, current treatment options for UC are limited and unable to induce
825 remission in all patients. Given the inflammatory nature of this disease, most treatments
826 entail immunosuppression. First line treatments, typically sulfasalazine and 5-
827 aminosalicylates including mesalamine, olsalazine, and balsalazide, induce remission in
828 about 50% of patients [16,248]. If 5-aminosalicylate therapy fails, patients with milder UC
829 may be prescribed oral glucocorticoids or immunosuppressives [249]. Azathioprine [250], 6-
830 mercaptopurine [251], and monoclonal antibody inhibitors of TNF-α, including infliximab
831 [252] and adalimumab [253,254], have all shown efficacy as immunosuppressives for UC. In
832 more severe cases, patients may receive intravenous glucocorticoids or cyclosporine to
833 attempt to induce remission [249,255]. Maintenance therapy during remission may include
834 oral or rectal 5-aminosalicylates or thiopurines, azathioprine, or 6-mercaptopurine.

835 Side effects of these treatments can be serious, including acute pancreatitis and
836 bone marrow suppression [256]. Patients unable to tolerate treatment or whose disease does
837 not respond to treatment may develop serious complications such as toxic megacolon,
838 bowel perforation, uncontrolled bleeding, and carcinoma or high-grade dysplasia, each of
839 which are indications for colectomy [257]. Unlike CD, colectomy is often curative for UC.
840 However, as many as 40-50% of patients develop pouchitis, whereby the artificial rectum
841 surgically created from ileal tissue after colectomy becomes inflamed [258]. Indeed, pouch
842 failure is estimated to occur in 4-10% of patients [16,259]. This inflammatory condition is

843 thought to result from changes in the microbiota within the ileal pouch, but the disease
844 mechanism is still unclear [259]. These side effects and the often limited effectiveness of
845 current treatments means novel treatments for UC are needed.
846

847 **4) Ulcerative Colitis and Fecal Microbiota Transplant**

848 Given that UC is thought to stem from dysbiosis and aberrant immune responses
849 to the microbiota, there was early interest in the use of probiotics and FMT to treat UC.
850 However, the mechanisms by which FMT may ameliorate UC are unknown, and FMT
851 use as adjunctive therapy remains controversial [260–262]. Following FMT for IBD, patients
852 exhibit microbiome compositions that resemble those of their donors [18,263–265]. A recent
853 randomized clinical trial comparing the efficacy of FMT versus water enema control
854 found a significant difference in levels of remission between the two groups, with 24% of
855 FMT-treated patients achieving clinical remission [18]. However, another randomized
856 clinical trial published the same year found no statistically significant difference in
857 remission rates between patients who received FMT from a healthy donor (41%
858 remission) and control patients who received FMT using their own feces (25% remission)
859 [265]. A recent meta-analysis of case-series studies of FMT for IBD showed that 45% of
860 patients achieved clinical remission following treatment, with higher rates of remission
861 observed for CD patients than for UC patients [266]. More research is needed to determine
862 why some IBD patients receiving FMT experience remission [263], while others have no
863 change in symptoms despite alterations in their gut microbiomes [264]. Identification of
864 microbial taxa that are associated with remission in patients who respond to FMT
865 treatment could result in development of more targeted probiotic therapeutics with
866 greater efficacy.

867

868 **5) Clinical Trials of Probiotics and UC**

869 Although this field is still in its infancy, recent clinical trials and meta-analyses
870 suggest that probiotics may be a viable option for adjuvant therapy in some UC patients
871 (Table 3). A recent systematic review of clinical trials evaluating probiotics for the
872 treatment of IBD concluded that although there was no evidence to suggest benefit in CD
873 treatment, probiotics and prebiotics were useful in helping to induce and maintain
874 remission of UC [13]. Twenty-one trials using probiotics for UC treatment were identified
875 in this review, with most considering either *E. coli* Nissle 1917 or the probiotic cocktail
876 VSL#3. One double blind double dummy study showed *E. coli* Nissle 1917 therapy to be
877 as effective as mesalamine in maintaining remission [14]. Another study showed
878 significantly greater induction of remission among pediatric UC patients treated with
879 VSL#3 compared to placebo-treated controls [15]. A few smaller scale studies showed other
880 probiotics, including BIO-THREE (*Enterococcus faecalis, Clostridium butyricum*, and
881 *Bacillus mesentericus*) [267] and *Bifidobacterium breve* [268], to also reduce disease activity.
882 Thus although there is still a paucity of well-designed, large-scale randomized controlled
883 trials, there is the potential that microbial therapy could serve as a viable alternative to
884 pharmacological therapy for UC.

885

886 **6) Protective Mechanisms of Probiotics against Ulcerative Colitis**

887 As discussed above, UC is characterized by the aberrant activation of the
888 inflammatory immune response. Epithelial barrier breakdown and subsequent increased
889 exposure to microbial products further stimulates immune responses and host immune-

890 induced epithelial damage. The ability of probiotics to promote epithelial barrier integrity
891 either directly by influencing junctional complexes or indirectly by affecting the cytokine
892 milieu and immune cell activation will thus likely have profound effects on UC disease
893 severity by limiting exposure to inflammatory signals and repairing host-induced
894 epithelial damage. Probiotics able to influence antigen presenting cell (APC) activation,
895 as well as downstream recruitment of effector immune cells, may also modulate immune
896 cell responses to inflammatory signals. There are thus multiple mechanisms through
897 which probiotics may limit the inflammation and barrier disruption observed in UC
898 pathogenesis. Some of these immunomodulatory mechanisms discovered for specific
899 probiotics that will be of potential interest in the design of probiotic cocktail therapy for
900 UC are discussed below.
901

902 ***1. Maintenance of the microbiota***

903 The ability of probiotic strains to restore or maintain the composition of the
904 microbiota may help prevent inflammatory immune signaling induced by dysbiosis.
905 Probiotic administration has been shown to limit dysbiosis in many disease states,
906 including colitis models, and to more quickly restore a normal microbiota composition
907 relative to placebo [269–271]. Patients with minimal hepatic encephalopathy treated with
908 LGG showed significantly increased Lachnospiraceae and Clostridia cluster XIV and
909 decreased Enterobacteriaceae and Porphyromonadaceae relative to placebo-treated
910 controls [272]. LGG has also been shown to prevent the increase in *Alicaligene* and
911 *Corynebacterium* species observed with chronic alcohol feeding in mice [273]. Similarly, *S.*
912 *boulardii* treatment in a diabetic mouse model led to increased Bacteroidetes and

913 decreased Firmicutes closer to levels observed in normal mice [274]. Administration of *S.*
914 *boulardii* to antibiotic-treated mice also resulted in a faster return to pre-antibiotic levels
915 of specific bacterial strains such as increased *Clostridium coccoides–Eubacterium rectale*
916 group members, including butyrate producers, and decreased *Enterobacteriaceae* and
917 *Bacteroides* species [271]. Restoration of butyrate producers may be especially helpful in
918 the context of UC, which is associated with decreased butyrate-producing bacteria [208].

919 Probiotics can also modulate the metabolic profile of the microbiota, suggesting a
920 means by which these organisms may help prevent alterations in the microbiota and limit
921 dysbiosis. For example, LGG administration to healthy 65-80 year olds induced no
922 change in overall microbiota composition as determined by 16S rRNA sequencing, with a
923 few exceptions such as increased butyrate producers *Roseburia* and *Eubacterium* [269].
924 However, expression of genes involved in bacterial motility and chemotaxis were
925 increased in certain commensal species, including *Bifidobacterium*, leading to the
926 suggestion that LGG can promote interactions between certain microbes and the host
927 epithelium [269]. More research is needed to determine if the effects of probiotic organisms
928 on microbiota composition and metabolic activity would confer protection in the context
929 of human UC.

930
931

932 ***2. Maintenance of intestinal epithelial integrity and barrier function***

933 Reinforcing the damaged GI epithelial barrier is a further potential avenue by
934 which probiotics may limit inflammatory responses in UC patients. As discussed above
935 for CDI, specific probiotics can directly influence the expression level, composition, and
936 organization of the mucus layer and junctional complex components. However, a key

feature of barrier dysfunction in UC is immune-mediated dysregulation of epithelial junctions via inflammatory cytokines, providing another avenue by which probiotic organisms may limit damage associated with UC (Table 2).

Inflammatory cytokines such as TNF-α, IFN-γ, and IL-23 are known to increase epithelial barrier breakdown and can be modulated by probiotic strains [275,276]. Several probiotic strains have been reported to downregulate TNF-α and IFN-γ production in mouse models of colitis, including *Lactobacillus brevis* SBC 8803, *Lactobacillus fermentum*, *Lactobacillus salivarius* subsp. *salivaris*, *Bifidobacterium lactis* [223], and mixtures of *Lactobacillus* and *Bifidobacterium* species [223,277]. *S. boulardii* also decreases TNF-α expression in mice [171]. Infectious models also demonstrate the ability of probiotics to limit GI inflammatory cytokine secretion, with LGG for example partially preventing the ETEC-induced increase in IPEC-J2 cell TNF-α expression [154]. Although the mechanisms underlying these probiotic-mediated decreases in inflammatory cytokine levels and associated barrier disruption are not well described, it is possible that probiotics act at least in part by modulating the overall cytokine milieu and inducing the production of anti-inflammatory cytokines.

3. Dampening inflammation through modulation of the cytokine milieu

The ability of probiotics to influence the cytokine milieu can have profound effects on disease severity by modulating the level of harmful host inflammatory immune responses. Anti-inflammatory cytokines IL-10 and TGF-β decrease the production of inflammatory cytokines, including IL-12p70, TNF-α, IL-1β, and IFN-γ [216]. In this manner, IL-10 and TGF-β are able to dampen host immune responses and limit

960 inflammation-mediated deregulation of barrier integrity [275,278]. Indeed, absence of IL-10
961 in mouse models significantly increases susceptibility to colitis, and IL-10
962 supplementation can ameliorate severity of chemically-induced colitis in mice [279,280].
963 Significantly, multiple probiotic species capable of ameliorating disease severity in colitis
964 models, including *Lactobacillus* species, *Bifidobacterium* species, and *E. coli*, increase
965 production of the anti-inflammatory cytokine IL-10 [281–286], and decrease expression of
966 inflammatory cytokines TNF-α [223] and IFN-γ [223,277].

967 Given the key role of APCs in directing the balance of the cytokine milieu,
968 numerous studies have screened probiotics for potential effectiveness in colitis based on
969 the ratio of inflammatory to anti-inflammatory cytokines they induce from APCs (Table
970 4). Indeed, the level of APC activation and cytokine production induced by different
971 probiotic species varies significantly [287], and there are reports that some probiotic strains
972 actually inhibit the effects of more stimulatory strains [287,288]. For example, addition of the
973 weak inflammatory cytokine inducer *Lactobacillus reuteri* with *L. casei* prevented the
974 previously noted high levels of activation markers (major histocompatibility complex
975 (MHC) II and CD86) and inflammatory cytokines (IL-12, IL-6, and TNF-α) induced by
976 *L. casei*, although IL-10 levels were not affected [287]. Thus, it is possible that addition of
977 particular strains may diminish potential beneficial effects of other strains in probiotic
978 cocktails. Some probiotics have also been reported to induce expression of APC
979 activation markers while simultaneously limiting cell activation in response to
980 inflammatory stimuli. For example, while one study reported strong CD80, CD86, and
981 CCR7 upregulation in human APCs exposed to *S. boulardii* [289], previous studies found *S.*
982 *boulardii* to inhibit lipopolysaccharide-induced upregulation of CD40, CD80, and CCR7

983 expression in human myeloid cells *in vitro* [290]. It will be important to assess specific
984 inhibitory properties of individual probiotic organisms on mucosal APCs, which are
985 known to have vastly different phenotypic profiles than *in vitro* bone marrow- or
986 monocyte-derived phagocytic cells, in order to more accurately assess whether these
987 probiotics may have beneficial effects in the context of UC.
988

989 ***4. Effects on neutrophil infiltration and function***

990 Given the clear association of UC pathology with neutrophil accumulation, the
991 ability of probiotics to regulate the recruitment, function, or apoptosis of neutrophils has
992 the potential to greatly influence the disease course (Table 4). Beneficial effects of
993 probiotics in limiting neutrophil-associated damage may stem from their ability to
994 modulate production of neutrophil chemotaxins and activators such as IL-17 and IL-8 [291–
995 296].

996 Probiotics may affect IL-17 levels by modulating expression of cytokines that
997 promote Th17 responses. Numerous probiotics have been found to downregulate IL-17
998 production and alleviate colitis, including *B. breve* [297], *B. longum* [298], *L. acidophilus* [299],
999 *B. longum* subsp. *infantis* [284], and *Streptococcus thermophilus* ST28 [300]. Additional
1000 studies have demonstrated the ability of several probiotic species to reduce the expression
1001 of Th17-promoting cytokines IL-6 and IL-23 [223] *in vitro* as well as in mouse models of
1002 liver fibrosis and GI permeability [171] and colitis [301–303]. Further mechanisms of probiotic
1003 modulation of Th17 cell differentiation may include inhibition of co-stimulatory
1004 molecules CD40 and CD80 on intestinal epithelial cells or downregulation of Th17-
1005 promoting transcription factors RORγt, STAT3, and NF-κB [223].

1006 Probiotics may also inhibit neutrophil-associated damage through modulation of
1007 IL-8. For example, *S. boulardii* was shown to produce a soluble factor that can inhibit
1008 NF-κB-mediated IL-8 production in IL-1β- and TNF-α-stimulated HT29 cells [294].
1009 Another study found *S. boulardii* to decrease IL-8 expression and neutrophil
1010 transmigration during infection of T-84 monolayers with *Shigella flexneri*, possibly by
1011 decreasing ERK, NF-κB, and JNK signaling [173]. Certain probiotics also alter neutrophil
1012 function, such as LGG inhibiting the formation of neutrophil extracellular traps formed in
1013 response to *Staphylococcus aureus* and phorbol 12-myristate 13-acetate (PMA)
1014 stimulation of *in vitro* human and murine neutrophils [304]. Thus, although more studies are
1015 needed to determine the temporal effects of probiotics on neutrophils in colitis models,
1016 modulation of neutrophil recruitment and activity is one clear way in which certain
1017 probiotics could ameliorate symptoms of UC.
1018

1019 **7) Summary of Probiotic Mechanisms of Action in UC and Implications for**
1020 **Future Therapies**

1021 UC is characterized by dysbiosis, GI barrier breakdown, and aberrant
1022 inflammatory immune responses, as described above (Fig 4.5). Particular microorganisms
1023 able to ameliorate any of these disease components could be of potential benefit in a
1024 combination microbial therapy designed for UC.

1025 As the exact species within the microbiota responsible for inciting disease
1026 pathology in UC are not well understood, it is only possible at present to identify
1027 probiotics that promote a microbiota associated with health and remission as opposed to
1028 active UC. It is thus of particular interest that probiotics such as LGG and *S. boulardii*

1029 have been found in some animal models to decrease levels of Enterobacteriaceae and
1030 Porphyromonadaceae, which are increased in active UC [272]. The ability of these and other
1031 probiotics to speed restoration of the microbiota following antibiotic treatment and to
1032 promote the activity of butyrate-producing bacteria may also help prevent insults that
1033 lead to active UC. Further studies are needed to determine the effects of particular
1034 probiotics in maintaining or restoring the microbiota specifically in the context of colitis.

1035 Also as discussed in the sections above on CDI, numerous probiotics are capable
1036 of reinforcing epithelial cell barrier function in the context of dysbiosis and
1037 inflammation. Strains able to prevent TNF-α-induced dysregulation of tight junctions,
1038 such as *L. plantarum* (strain L2) [175] and *B. bifidum* (strains WU12, 20, and 57) [174], may
1039 be of particular benefit in UC where levels of this inflammatory cytokine are elevated [217].
1040 Reinforcement of barrier integrity may help to reduce immune stimulation and prevent
1041 exacerbation of inflammatory responses to microbial antigens.

1042 It is important to note when considering these studies (Table 4) that most relied on
1043 *in vitro* models to evaluate cytokine induction by probiotics. Effect of probiotics on the
1044 many other immune cells present in the GI mucosa may lead to strikingly different
1045 consequences *in vivo* than would be predicted from *in vitro* studies. Furthermore,
1046 particular effects of probiotics may depend on the composition of the endogenous
1047 microbiota, whose composition is greatly altered in the context of UC, antibiotic
1048 treatment, and CDI. More *in vivo* studies are clearly needed to determine the exact effects
1049 that probiotic strains will have on cytokine induction in the context of particular diseases
1050 such as UC and CDI.

1051 Given that the roles of particular immune cell subsets in UC pathogenesis are still
1052 incompletely understood, predicting which probiotics might ameliorate disease symptoms
1053 based on their immunological effects becomes a difficult task. Furthermore, particular
1054 probiotics may still have beneficial effects *in vivo* despite inducing what might be
1055 considered counterproductive effects on specific immune cell subsets *in vitro*. *E. coli*
1056 Nissle 1917, for example, has been shown in clinical trials to help mitigate UC [14,305,306],
1057 yet induces secretion of the neutrophil chemoattractant IL-8 *in vitro* [291]. Such findings
1058 highlight the importance of studying the effects of particular probiotic strains on the
1059 epithelium and microbiota as well as on immune cells *in vivo* as the relative importance
1060 of each mechanism for individual probiotic strains may differ.
1061

1062 **d) Discussion of Therapeutic Uses of Probiotics**

1063 FMT is a promising but as yet unrefined therapy for many infectious and
1064 autoimmune GI disorders. Both recurrent CDI and UC can be successfully treated with
1065 FMT, but the microbial components responsible for benefit are still unknown. Identifying
1066 particular strains that confer protection in each case would allow for design of combined
1067 microbial therapies to treat disease while eliminating risks associated with the transfer of
1068 unknown components of the microbiota from human donors.
1069 The ability to create such tailored therapy will require a thorough understanding
1070 both of disease pathogenesis and the *in vivo* mechanisms of action of particular beneficial
1071 microbial strains and combinations of strains. In many cases, an incomplete
1072 understanding of the roles played by host and microbial cells will limit the ability to
1073 predict effective therapy. Better tailored microbial therapies for CDI and UC may in fact

1074 become possible as further studies continue to clarify the roles played by the microbiota
1075 and by host cells, leading to novel targets. Still, available data regarding the actions of
1076 particular beneficial microbial strains, viewed in light of the current understanding of
1077 CDI and UC pathogenesis, allows for identification of candidate probiotic strains for
1078 further testing (Tables 2 and 4).

1079 Based on the available evidence of probiotic mechanisms of action, it seems
1080 unlikely that individual probiotic strains would confer the full repertoire of benefits
1081 necessary for protection against disease. Indeed, most clinical trials considering single
1082 probiotic strains for treatment of CDI have found no benefit (Table 1). Combinations of
1083 strains with complementary actions targeting a variety of factors involved in disease may
1084 instead be much more likely to confer protection against disease. General categories of
1085 action may include effects on the composition of the microbiota, host epithelial barrier
1086 integrity, and immune responses.

1087 Unfortunately, *in vitro* studies suggest that optimal combined therapies may not
1088 always be predictable based on studies of individual strains [287]. Indeed, it is possible that
1089 some probiotics will have inhibitory effects on other co-administered strains and limit
1090 overall efficacy. Thus, although the many studies of individual probiotic strains provide
1091 useful information regarding potential mechanisms of action and identify candidates for
1092 therapy, further experiments will be necessary to determine if beneficial effects are
1093 maintained in combination with other probiotic strains *in vivo*.

1094 Finally, although this review focuses on CDI and UC as examples, general
1095 principles regarding probiotic mechanisms of action can also be applied to similar GI
1096 pathogens and other forms of colitis, including CD. Indeed, effects of probiotics on

1097 reinforcing the epithelial barrier and limiting immune cell inflammation may be highly
1098 beneficial in ameliorating symptoms of many GI diseases. Reinforcement of the GI
1099 barrier may help protect the host from increased exposure to either specific toxins, as in
1100 the case of CDI or other infections, or to components of the microbiota inciting
1101 autoimmune inflammation, as in the case of colitis. Ability to direct immune responses
1102 against pathogens or to limit aberrant inflammatory responses are additional clear ways in
1103 which probiotics could help to ameliorate disease. However, further studies are needed to
1104 specifically determine the effects of particular probiotic strains *in vivo* in the context of
1105 each disease.

1106 Given the rising incidence of CDI and UC worldwide, improved therapies for
1107 these serious GI conditions are urgently needed. FMT and a few select probiotics already
1108 provide some benefit in preventing and treating these diseases. Improving microbial
1109 therapies through use of defined combinations of beneficial strains tailored to each
1110 disease holds significant promise for expanding this line of adjuvant therapy and
1111 revolutionizing treatment of these diseases. The information in this review will help to
1112 direct future studies of probiotic efficacy and speed the development of these much-
1113 needed treatments.
1114

1115 **e) Novel Applications of Probiotics**

1116 The innate properties of probiotic organisms described above hold great potential
1117 for the design of effective adjuvant therapies for many gastrointestinal disorders such as
1118 CDI and UC. An intriguing novel application of probiotics, however, involves extending
1119 use of these organisms to synthesize and deliver recombinant therapeutics directly to the

1120 gastrointestinal tract. Transforming probiotics to administer oral therapeutics or vaccines
1121 could have significant economic and immunological advantages over current standard
1122 therapies. A simultaneous synthesis and delivery system involving mass production of
1123 therapeutics by probiotic microorganisms would significantly reduce the expense
1124 currently associated with therapeutic synthesis and encapsulation into particles resistant
1125 to *in vivo* degradation. Targeting therapeutics directly to the intestinal tract would also
1126 enable modulation of local mucosal immune responses, which are not as effectively
1127 stimulated by systemic administration [307]. Indeed, several experimental systems have
1128 successfully demonstrated the potential for some probiotics to synthesize and deliver
1129 vaccine antigens and therapeutics to the gastrointestinal tract. Recombinant probiotic
1130 bacteria, particularly lactic acid bacteria, have been shown to induce protective mucosal
1131 immune responses against numerous viral and bacterial pathogens as mucosal vaccine
1132 delivery vectors, demonstrating feasibility of this vaccination strategy [308].

1133 The probiotic yeast *Saccharomyces boulardii* has several characteristics that may
1134 confer potential advantages over use of prokaryotic probiotic strains. As a eukaryote, *S.*
1135 *boulardii* is capable of post-translational modifications that may permit synthesis of
1136 complex antigens in a conformation closer to their native state. This may be especially
1137 advantageous for expression of complex antigens such as those found on intestinal
1138 parasites, including helminths. In addition *S. boulardii* would be less likely than bacterial
1139 strains to exchange recombinant DNA with other resident microbiota. Unlike most
1140 probiotic bacterial strains, *S. boulardii* is not a natural colonizer of the gut in either
1141 humans or mice, avoiding prolonged intestinal exposure to vaccine antigens that may
1142 increase the risk of tolerance induction.

1143 *S. boulardii* also possesses a number of key characteristics distinguishing it from
1144 closely related *S. cerevisiae* strains including faster growth rate, increased resistance to
1145 stresses within the gastrointestinal tract, and status as a Generally Recognized as Safe
1146 (GRAS) microorganism [309,310]. Indeed, numerous clinical trials have evaluated efficacy of
1147 *S. boulardii* in treating not only CDI and UC but other gastrointestinal diseases such as
1148 acute diarrhea and travelers' diarrhea, as described extensively elsewhere [311–314]. Current
1149 clinical use of *S. boulardii* may help to facilitate further applications of *S. boulardii* as a
1150 therapeutic or vaccine delivery vector. Here is presented a brief overview of the current
1151 understanding of *S. boulardii*: its phylogenetic classification, characteristics of
1152 gastrointestinal transit, interactions with host immune cells, and potential for expression
1153 of heterologous proteins. Particular emphasis is placed on those features that may
1154 influence function as a vaccine delivery system and that will provide context for the
1155 following chapters which further evaluate and develop *S. boulardii* for this application.

1156 **f) Phylogenetic Classification of *S. boulardii***

1157 The first official description of *S. boulardii* as a probiotic was in 1982 [315,316].
1158 Although there has been much debate as to the classification of this organism, current
1159 consensus is that *S. boulardii* is a subspecies of the well-studied budding yeast
1160 *Saccharomyces cerevisiae.* Early studies using rRNA sequencing and PCR
1161 electrophoretic karyotyping found that *S. boulardii* could not be differentiated from *S.*
1162 *cerevisiae* [276]. Recent genetic analyses, including use of microsatellite polymorphism
1163 analysis and retrotransposon analysis have demonstrated distinct clustering of *S.*
1164 *boulardii* from various *S. cerevisiae* strains [317,318], offering support for the current
1165 understanding of *S. boulardii* as a subspecies of *S. cerevisiae*. This was confirmed in a

1166 comparative genome hybridization approach evaluating DNA/DNA hybridizations for all
1167 *S. cerevisiae* open reading frames (ORFs) [319,320]. Furthermore, Fietto *et al.* used PCR
1168 targeting intron sequences, a common method to distinguish between commercial yeast
1169 strains, and found two bands they suggested could be used to distinguish *S. boulardii*
1170 from strains of *S. cerevisiae* [321]. It is important to consider, however, that the estimated
1171 95% homology between *S. boulardii* and reference *S. cerevisiae* strains [322] still allows for
1172 numerous distinct characteristics of the *S. boulardii* subspecies [323].
1173

1174 g) *S. boulardii* Stress Resistance and Kinetics of Gastrointestinal 1175 Transit

1176 A number of characteristics distinguishing *S. boulardii* from other strains of *S.*
1177 *cerevisiae* may provide advantages for use within the intestine. For example, *S. boulardii*
1178 has been reported to have increased growth rates relative to *S. cerevisiae* strains,
1179 including increased growth at higher temperatures (37°C versus 30°C) [309,310]. Wild type S
1180 *boulardii* also demonstrated increased resistance to a wide range of pH and bile acid *in*
1181 *vitro* relative to *S. cerevisiae*, reflecting the potential to withstand stresses within the
1182 gastrointestinal tract [309,321]. Indeed, relatively high percentages of viable *S. boulardii*
1183 were recovered after short term incubation within the mouse intestine [309].
1184 Furthermore, although *S. boulardii* is not a natural colonizer of the
1185 gastrointestinal tract in humans or mice [324–326] some studies have demonstrated
1186 differences in survival and rate of clearance from the gastrointestinal tract for *S. boulardii*
1187 and *S. cerevisiae*. In gnotobiotic mice, *S. cerevisiae* is cleared from the intestine in less
1188 than 24 hours whereas *S. boulardii* can be detected in the stool for ten days post gavage

1189 [137]. Comparison of *S. boulardii* and three probiotic bacterial strains demonstrated that
1190 *Bifidobacterium animalis* var. *lactis* BB-12 and *Escherichia coli* EMO presented the
1191 highest values of colonization (10^{10} CFU/g feces) in gavaged gnotobiotic mice, while
1192 *Lactobacillus casei* and *S. boulardii* values fluctuated between 10^4 and 10^7 CFU/g feces
1193 during a 10 day period [136]. Comparison of *S. boulardii* and *S. cerevisiae* strains Σ1278b
1194 and BY3 in the gastrointestinal tract of SPF mice found all three strains to be excreted
1195 from the gut within 24 hours, with most cells excreted between 3 to 6 hours post gavage
1196 [309]. Percent recovery of yeast from the cecum and colon was greater than from the
1197 stomach and small intestine after only one hour post gavage. Furthermore, they noted
1198 significant within-group variability for yeast recovery and kinetics, preventing the ability
1199 to detect any significant differences between the yeast strains.

1200 In a human study, steady state fecal concentrations of *S. boulardii* ($2x10^7$ CFU/g
1201 feces) were achieved by 3 consecutive days of administration in eight healthy volunteers
1202 [327], and yeast were cleared from feces four days after discontinuation. Additional studies
1203 found recovery of live yeast from feces to be <1%[326] and <5% [328] of the initial inoculum
1204 in humans and rats. Klein *et al.* also noted that human volunteers treated with ampicillin
1205 had *S. boulardii* steady state levels two to three times higher than volunteers not treated
1206 with antibiotics [328]. Such an effect suggests that *S. boulardii* is normally limited by the
1207 intestinal microbiota, which also has implications for administration of *S. boulardii* to
1208 immunocompromised individuals or people receiving antibiotic therapy. Overall, these
1209 findings indicate that *S. boulardii* is capable of traversing the intestine of humans and
1210 rodents, but that it is unable to persist. *S. boulardii* thus has the potential to deliver

heterologous antigen along the intestine without prolonged exposure that might result from intestinal colonization.

h) Interactions of *S. boulardii* with Host Immune Cells

Although described above in the context of its use as a probiotic for treatment of CDI and UC (Tables 4.2 and 4.4), the potential immunomodulatory effects of *S. boulardii* may also influence its efficacy as vaccine delivery vector. *S. boulardii* modulation of the cytokine balance, immune cell differentiation, or antibody induction may all affect the nature of immune responses to vaccine antigen. It is thus vital to consider the known effects of *S. boulardii* on the mucosal immune system in order to understand the potential influences of the vector itself on vaccine responses. Below is a brief overview of some of the known immunomodulatory effects of *S. boulardii* that may impact its novel role as a vaccine delivery vector.

1) *S. boulardii* and the Cytokine Milieu

The balance of cytokines induced by *S. boulardii* may affect the both the degree and types of immune responses against *S. boulardii* expressed vaccine antigens. Several studies have shown *S. boulardii* to promote production of the anti-inflammatory cytokine IL-10 in the context of inflammation. For example, administration of either viable or heat-killed *S. boulardii* induced increased serum IL-10 levels without affecting levels of IFN-γ in a colitis model [138]. Another study found that *S. boulardii* induced significantly increased serum IL-10 levels in a murine intestinal obstruction model [136]. Induction of such anti-inflammatory cytokines as IL-10 may limit immune responses to vaccine

1233 antigen and necessitate co-administration of adjuvant to overcome induction of tolerance
1234 to vaccine antigens.

1235 In contrast, influencing the production of particular inflammatory cytokines may
1236 influence differentiation of T cell subsets and lead to greater vaccine efficacy in the
1237 context of some diseases. Some experiments for example have demonstrated that levels
1238 of the inflammatory cytokines TNF-α, IL-12, and IFN-γ peaked earlier and at higher
1239 levels in germ free mice infected with *E. coli* B_{41} and fed S *boulardii* relative to unfed
1240 infected mice [137]. Another study also showed that *S. boulardii* coincubation prevented the
1241 EHEC-induced IL-8 increase in T84 cells [329]. Thus the particular cytokines induced by *S.*
1242 *boulardii*, as well as potential downstream effects on immune cell activation and
1243 differentiation, appear to vary depending on the disease in question.

1244 **2) *S. boulardii*-induced Antibody Production**

1245 Several studies have demonstrated induction of secretory IgA (sIgA) with oral
1246 administration of *S. boulardii*, particularly in monoassociated and neonatal rodent
1247 models. Studies have found increased total IgA with *S. boulardii* administration in
1248 neonatal rats [135], monoassociated germ free mice [136], and in a model of intestinal
1249 obstruction and *E. coli* challenge in mice [138]; however, these studies did not investigate
1250 specificity of the increased IgA. A later study found that *S. boulardii* increased both total
1251 sIgA and anti-*S. boulardii* sIgA in germ free mice and that germ free mice not fed *S.*
1252 *boulardii* also had low levels of IgA binding to S *boulardii* [137]. However, they found no
1253 change in either total or anti-*Saccharomyces* IgG. *S. boulardii* coadministration with *C.*
1254 *difficile* toxin was also found to increase both total and anti toxin A IgA antibody
1255 secretion in BALB/c mice [132].

1256 In sum, numerous studies have found increased IgA with *S. boulardii*
1257 administration in rodent models and some degree of antibody induced against either *S.*
1258 *boulardii* itself or other co-administered antigens. While induction of antibody responses
1259 may be useful in sequestration of pathogens and toxins within the intestine in the context
1260 of disease, increased antibody directed against *S. boulardii* may increase sequestration of
1261 the vaccine vector itself and prevent sufficient delivery of vaccine antigen to immune
1262 tissues. Antibody induction against *S. boulardii* should thus also be carefully evaluated in
1263 the context of vaccine delivery to ensure the yeast vector is not sequestered away from
1264 the intestinal mucosa.
1265

1266 i) Genetic manipulation and transformation of *S. boulardii*

1267 One hurdle to successful and efficient use of *S. boulardii* as a vaccine delivery
1268 vector is the optimization of transformation protocols. There have been several reports of
1269 successful transformation of WT *S. boulardii* [309,330–332]; however, to date few have
1270 specified the transformation method used and in at least one study it was suggested that *S.*
1271 *boulardii* has lower transformation efficiency relative to *S. cerevisiae* [330]. Transformation
1272 using LiOAc was adopted in two studies [316,331]. Electroporation has also been used to
1273 transform *S. boulardii* [332]. There have been no reports of transformation using
1274 spheroplasty, biolistic method, or glass bead approaches. Another study reported
1275 improved transformation efficiency and screening of *S. boulardii* using the commercially
1276 available Invitrogen *S.c*. EasyComp™ Transformation Kit [333]. Until recently, selection
1277 of transformants entailed use of antibiotics such as kanamycin and hygromycin to which

1278 *S. boulardii* is susceptible. Such reliance on antibiotic resistance markers for selection
1279 could pose a risk in the context of mass administration in vaccine delivery.
1280

1281 **j) *Saccharomyces* recombinant antigen expression**

1282 The ability of *S. boulardii*, as a eukaryotic organism, to potentially express
1283 complex, glycosylated antigens could serve as a significant advantage in its use as a
1284 vaccine delivery vector. Indeed, the closely related *S. cerevisiae* is already used to
1285 produce such compounds as insulin, hepatitis B surface antigen, granulocyte macrophage
1286 colony stimulating factor (GM-CSF), and platelet derived growth factor [334].
1287 Although genetic manipulation of *S. boulardii* is still in its infancy and successful
1288 *S. boulardii*-based vaccines have yet to be established, some studies have begun to
1289 explore other applications of *S. boulardii* as a therapeutic. One study has reported
1290 engineering *S. boulardii* to express the anti inflammatory cytokine IL-10 [331].
1291 Furthermore, the authors tested whether administration of this recombinant yeast could
1292 protect mice from DSS colitis relative to control yeast. After ten days of daily oral
1293 treatment with either control or IL-10-expressing yeast, there was no apparent difference
1294 in histological score or colonic thickening between yeast and control groups. However,
1295 both IL-10-expressing yeast and control yeast improved the ulceration score. Further
1296 studies are needed to explore the potential of using *S. boulardii* as a delivery vector for
1297 immune modulatory therapeutics.
1298

1299 **k) *Saccharomyces* experimental vaccines**

1300 Current experimental uses of *S. cerevisiae* as a delivery vector for vaccines
1301 suggests the potential of the closely related *S. boulardii* to successfully express highly
1302 complex heterologous antigens and induce protection against disease. Indeed, *S.*
1303 *cerevisiae* has been used to induce protective immune responses against a wide variety of
1304 cancers and infectious diseases [259]. In one study, *S. cerevisiae* expressing Ras generated
1305 antigen specific immune responses against Ras-expressing tumor cells [336]. Several studies
1306 have also found recombinant *S. cerevisiae* expressing carcinoembryonic antigen (CEA)
1307 to activate DCs and CEA-specific T cells *in vitro* as well as to decrease T regulatory cells
1308 and increase antigen specific CD4 and CD8 T cells in phase one clinical trials [337–339].
1309 Subcutaneous treatment with recombinant *S. cerevisiae* expressing a hepatitis C virus
1310 (HCV) nonstructural protein 3 (NS3)-core fusion protein was also found to promote
1311 elimination of HCV $NS3^+$ tumor cells in mice as well as a trend towards undetectable
1312 patient HCV loads in a phase two clinical trial[340,341]. Expression of heterologous antigen
1313 by *S. cerevisiae* is thus clearly able to induce specific, inflammatory immune responses
1314 either *in vitro* or via subcutaneous yeast delivery.

1315 Importantly, one group has also previously demonstrated successful use of
1316 transformed *S. cerevisiae* in an oral vaccine system. *S. cerevisiae* expressing the
1317 immunodominant ApxIIA antigen of *Actinobacillus pleuropneumoniae* (a pig respiratory
1318 pathogen) was orally administered to mice prior to challenge [342,343]. Increased antigen-
1319 specific IgA responses in the lung and small intestine and increased antigen-specific
1320 systemic IgG and IgM were detected in vaccinated mice. Vaccination was also found to
1321 decrease serum and lung concentrations of the inflammatory cytokines IL-1b, TNF-α, and

1322 IL-6 and increase survival post challenge with *A. pleuropneumoniae*. There is thus a
1323 precedent that a yeast strain can successfully be used to vaccinate mice and stimulate
1324 protective responses both within the intestine and lungs.
1325

1326
l) Oral Tolerance

1327 To induce antigen-specific protective immune responses, oral vaccines must
1328 overcome the obstacle of oral tolerance. This phenomenon describes the tendency of oral
1329 exposure to antigens to reduce subsequent immune responses specifically to that antigen,
1330 either in the intestine or systemically [344]. Development of oral tolerance is usually
1331 reflected in delayed-type hypersensitivity responses and decreased T cell proliferation,
1332 cytokine secretion, and serum antibody responses to antigen.
1333 Many cell types within the intestinal mucosa are thought to contribute to oral
1334 tolerance, although the exact mechanisms underlying this phenomenon are still not
1335 completely understood. The role of Peyer's patch (PP) microfold (M) cells in tolerance
1336 induction is not entirely clear: although some studies have reported increased tolerance
1337 with protein targeting to M cells [345], others have demonstrated induction of tolerance
1338 even in the absence of PPs [346,347]. Dendritic cells are known to be key players involved in
1339 the induction of oral tolerance by trafficking to the mesenteric lymph nodes (MLNs) and
1340 producing retinoic acid, which imprints the expression of gut homing markers on T and B
1341 cells [348]. $CD4^+$ $CD25^+$ T cells, including $Foxp3^+$ T regulatory cells (Tregs), are also
1342 known to be highly involved in the induction and maintenance of oral tolerance through
1343 the production of cytokines such as IL-10 and TGF-β [344]. The roles of many other cell

1344 types and interactions involved in oral tolerance have been reviewed extensively [344,349–
1345 351].

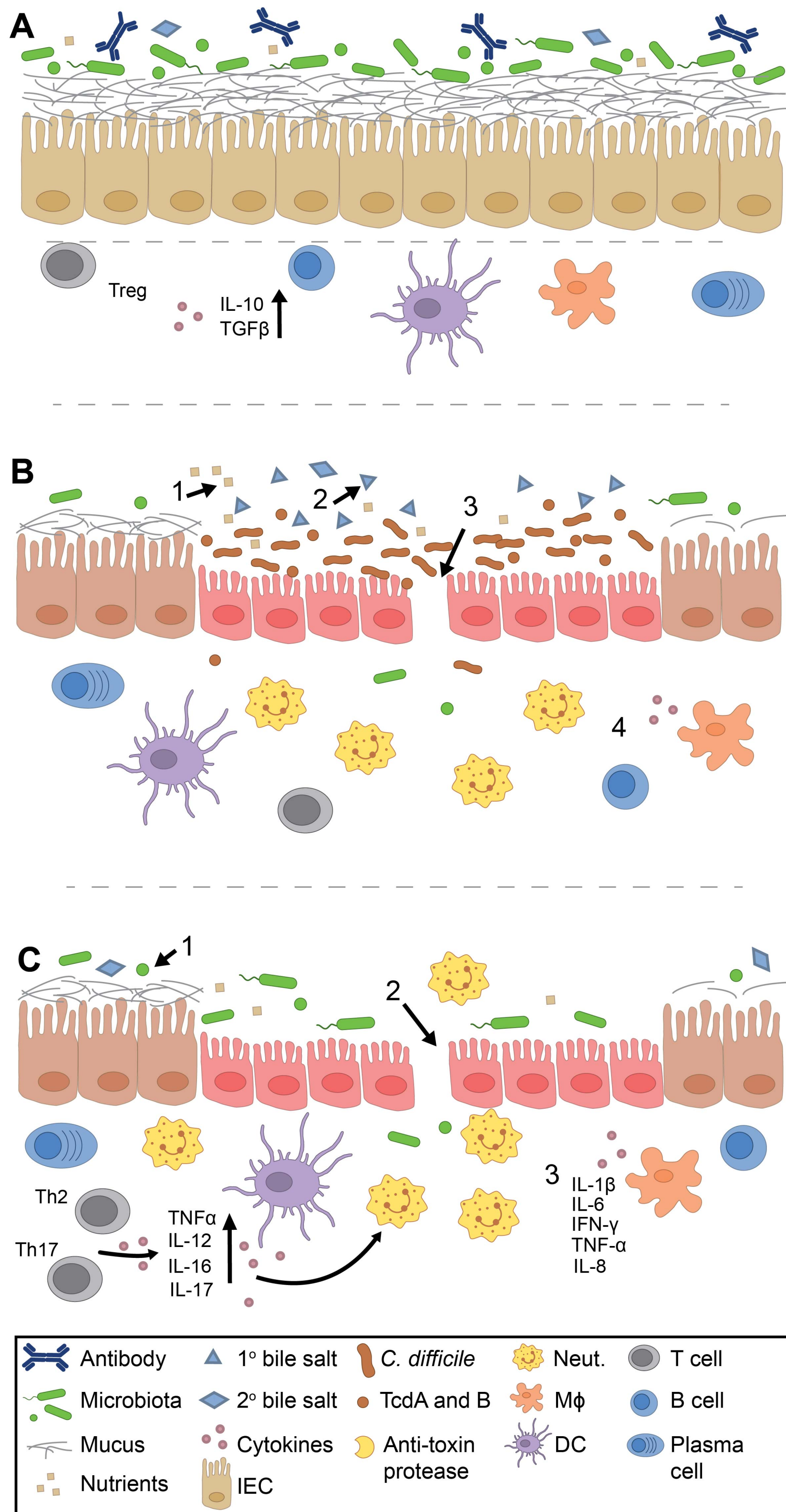
1346 Although an adaptive response to the many food antigens encountered within the
1347 gastrointestinal tract, oral tolerance may prevent the development of protective immune
1348 responses to mucosal vaccine antigen. To avoid this, numerous groups have adopted use
1349 of mucosal adjuvants such as cholera and *E. coli* toxins [352], cytokines [353], immune-
1350 stimulating complexes (ISCOMs) [307], and others in combination with oral vaccines.
1351 These compounds act through many different mechanisms such as by binding TLRs and
1352 are thought to serve as danger signals to promote the generation of inflammatory immune
1353 responses and avoid induction of tolerance to vaccine antigen. Selection of the optimal
1354 mucosal adjuvant for use in combination with an *S. boulardii*-based vaccine delivery
1355 system will be crucial for induction of antigen-specific protective rather than tolerogenic
1356 responses.
1357

1358 **m) Summary**

1359 Probiotic organisms may provide significant benefits as adjuvant therapy for
1360 gastrointestinal diseases such as CDI and UC. By increasing our understanding of the
1361 pathophysiology of these diseases and the beneficial mechanisms of action of specific
1362 probiotic strains, it may be possible to rationally design a combined microbial therapy
1363 that confers the benefits but not the complications sometimes associated with FMT.
1364 An additional proposed application of probiotics includes the use of specific
1365 strains to express recombinant therapies (Fig 4.6). As described above and in Chapters 6
1366 and 7, *S. boulardii* possesses a number of key characteristics that may be advantageous

1367 for the synthesis and delivery of oral therapeutics and vaccines. However, a few key
1368 studies are necessary before *S. boulardii* can be thoroughly evaluated for this application.
1369 First is the investigation of the interactions of *S. boulardii* with the healthy, uninflamed
1370 intestine. Although most studies of *S. boulardii* to date have been *in vitro* or in the
1371 context of an inflamed intestine, use of *S. boulardii* as a vaccine vector will entail
1372 delivery to a healthy gut. Chapter 6 thus describes interactions of *S. boulardii* with the
1373 healthy adult mouse intestine and the potential implications for vaccine design. Next, it is
1374 necessary to demonstrate that *S. boulardii* can safely and efficiently be transformed to
1375 express heterologous antigen. Wild type *S. boulardii* is selected using expensive and
1376 potentially hazardous antibiotics and resistance markers. Chapter 7 describes the
1377 generation of mutant strains of *S. boulardii* that can be easily manipulated using standard
1378 techniques and express heterologous protein without antibiotic selection. Pilot
1379 vaccination experiments described in Chapter 8 demonstrate successful expression of
1380 further antigens by this mutant strain of *S. boulardii* and provide the foundation for
1381 additional vaccination studies in mice. Each of these components are crucial first steps to
1382 characterize *S. boulardii* as an oral vaccine delivery vector and advance the development
1383 of this novel application of probiotic organisms.
1384

1385 **n) Figures and Tables**



1386

1387 **Fig 4.1 The gastrointestinal mucosa in health, CDI, and UC**

1388 (A) The healthy mucosa is characterized by a diverse microbiota that confers colonization
1389 resistance and proper immunomodulation; few freely available nutrients; low levels of
1390 and primary bile salts relative to secondary bile salts; secretory antibody capable of
1391 sequestering commensals, pathogens, and other antigens to prevent translocation; an
1392 intact barrier with healthy epithelial cells and thick layers of mucus containing
1393 antimicrobial peptides; few immune cells and a cytokine milieu dominated by anti-
1394 inflammatory cytokines such as IL-10 and TGFβ. (B) *C. difficile* growth is promoted by
1395 disruption of the microbiota, which results in (1) increased nutrients permissive for *C.*
1396 *difficile* growth and (2) high concentrations of primary bile salts relative to secondary bile
1397 salts. This leads to buildup of high concentrations of *C. difficile* and its toxins. (3) Toxins
1398 damage epithelial cytoskeletal components, leading to cell death and ulcerations. (C)
1399 Ulcerative colitis is characterized by (1) an altered microbiota of decreased diversity, (2)
1400 damage to the gastrointestinal epithelium, as well as (3) aberrant, overly inflammatory
1401 host immune responses. Abbreviations: IEC (intestinal epithelial cell), TcdA and TcdB
1402 (*C. difficile* toxins A and B), Neut. (neutrophil), Mϕ (macrophage), DC (dendritic cell).

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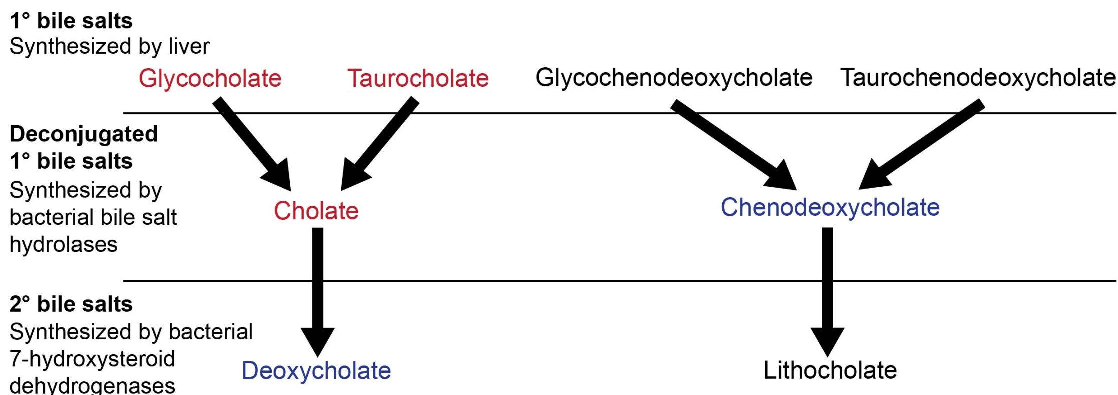


1405

1406

1407 **Fig 4.2 Summary of bile salt metabolism**

1408 Primary bile salts (1°) produced by the host liver are modified and deconjugated by

1409 intestinal bacteria to form secondary bile salts (2°). In red are bile salts that stimulate

1410 germination of *C. difficile* spores, thus increasing susceptibility to CDI. In blue are bile

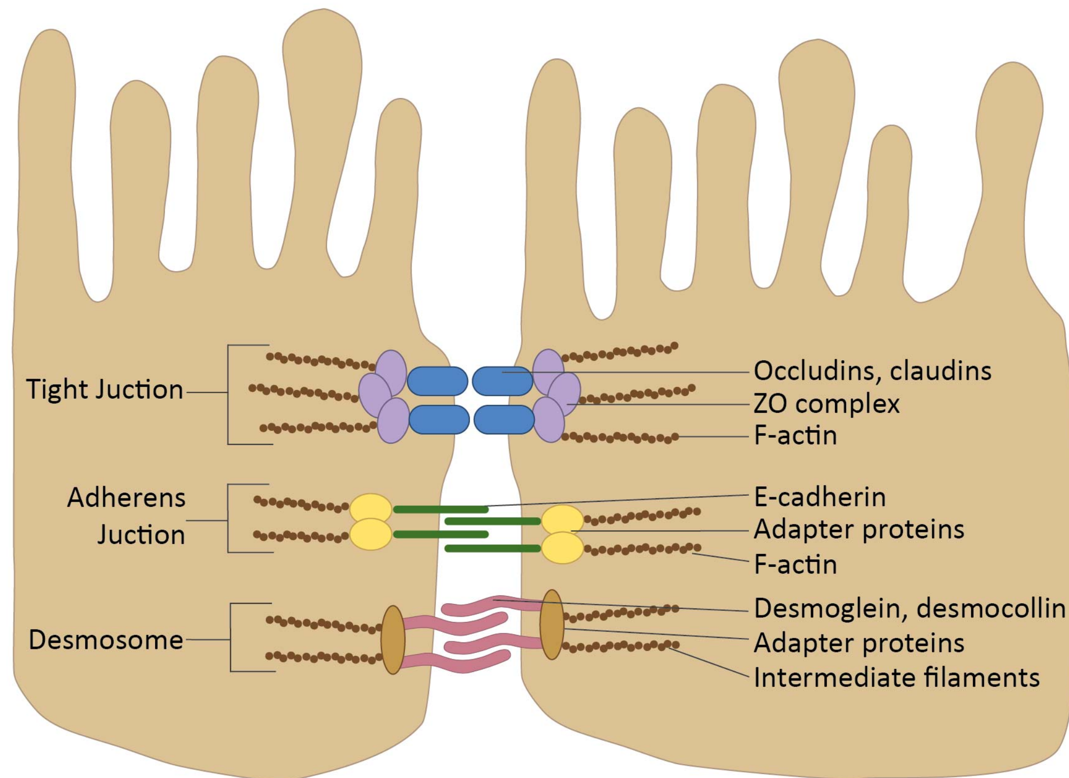
1411 salts known to inhibit sporulation or outgrowth of *C. difficile* and therefore contribute to

1412 colonization resistance. Probiotics with 7-hydroxysteroid dehydrogenase activity would

1413 decrease levels of glycocholate, taurocholate, and cholate in the intestines while

1414 increasing levels of deoxycholate, thereby enhancing colonization resistance.

1415

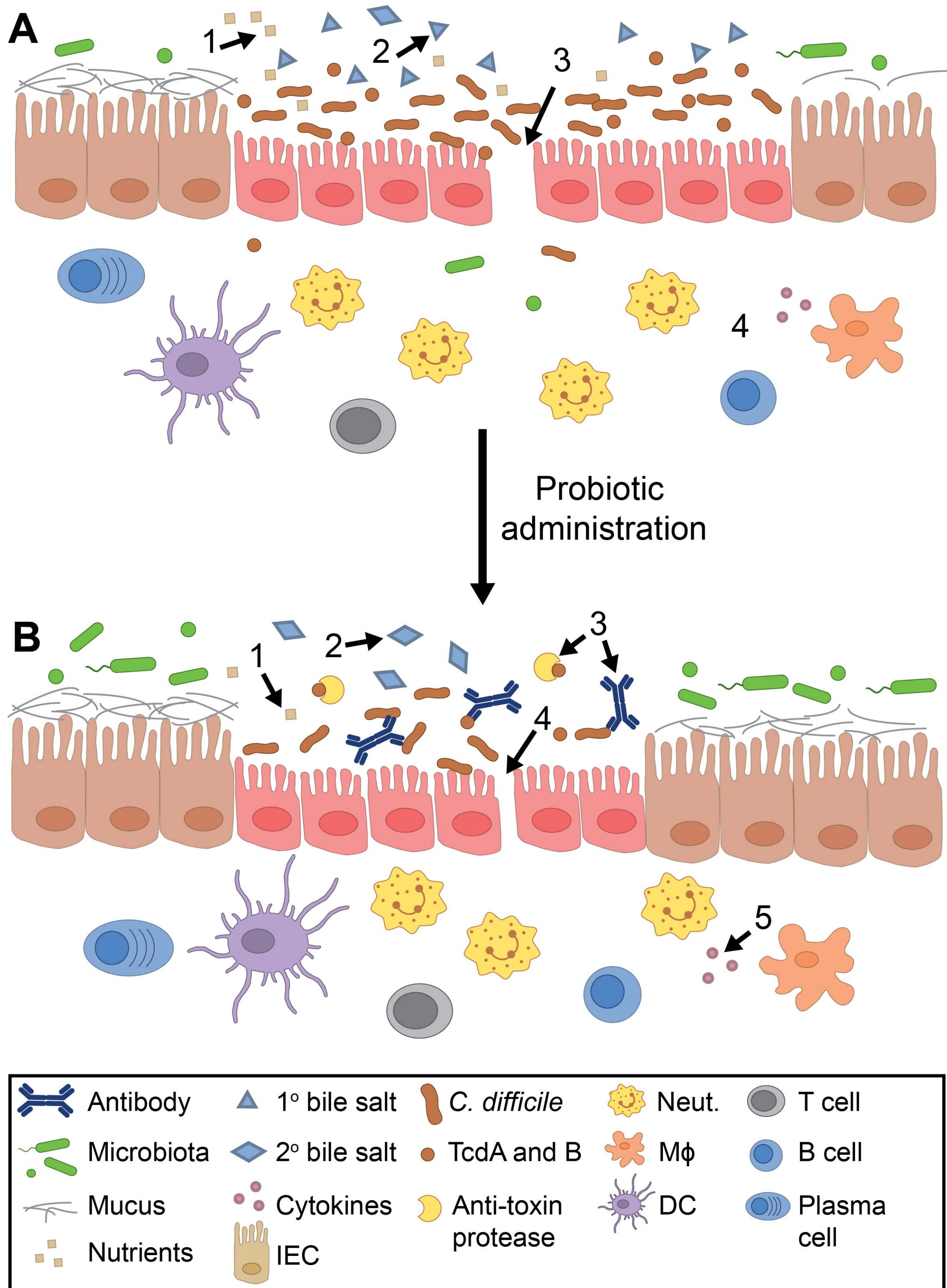


1416

1417 **Fig 4.3 Epithelial cell junctional complex**

1418 Adjacent epithelial cells are held tightly together by the junctional complex. Apically,
1419 tight junctions are composed of occludins and claudins that span the intercellular space
1420 and bind adapter proteins such as zonula occludens (ZO) complex proteins. Adherens
1421 junctions are composed of E-cadherins and adapter proteins, and desmosomes are formed
1422 of desmoglein and desmocollin that bind internal adapter proteins. The adapter proteins
1423 in each complex also bind components of the cytoskeleton, including F-actin or
1424 intermediate filaments.

1425



1426

1427 **Fig 4.4 Potential effects of probiotics in CDI**

1428 (A) *C. difficile* growth is promoted by disruption of the microbiota, which results in (1)
1429 increased nutrients permissive for *C. difficile* growth and (2) high concentrations of
1430 primary bile salts relative to secondary bile salts. This leads to buildup of high
1431 concentrations of *C. difficile* and its toxins. (3) Toxins damage epithelial cytoskeletal
1432 components, leading to cell death and ulcerations. (B) Probiotics may promote
1433 colonization resistance through (1) competition for nutrients and (2) generation of
1434 secondary bile salts. Probiotics may also directly inhibit the growth of *C. difficile* by
1435 producing bacteriocins or other inhibitory compounds. Some probiotics (3) produce anti-
1436 toxin proteases and may stimulate antibody production to sequester *C. difficile* and toxin.
1437 (4) Reinforcement of the epithelial barriers and (5) modulating inflammatory host
1438 responses may also promote healing and decrease damaging host responses to infection.
1439 Abbreviations: IEC (intestinal epithelial cell), TcdA and TcdB (*C. difficile* toxins A and
1440 B), Neut. (neutrophil), Mϕ (macrophage), DC (dendritic cell).
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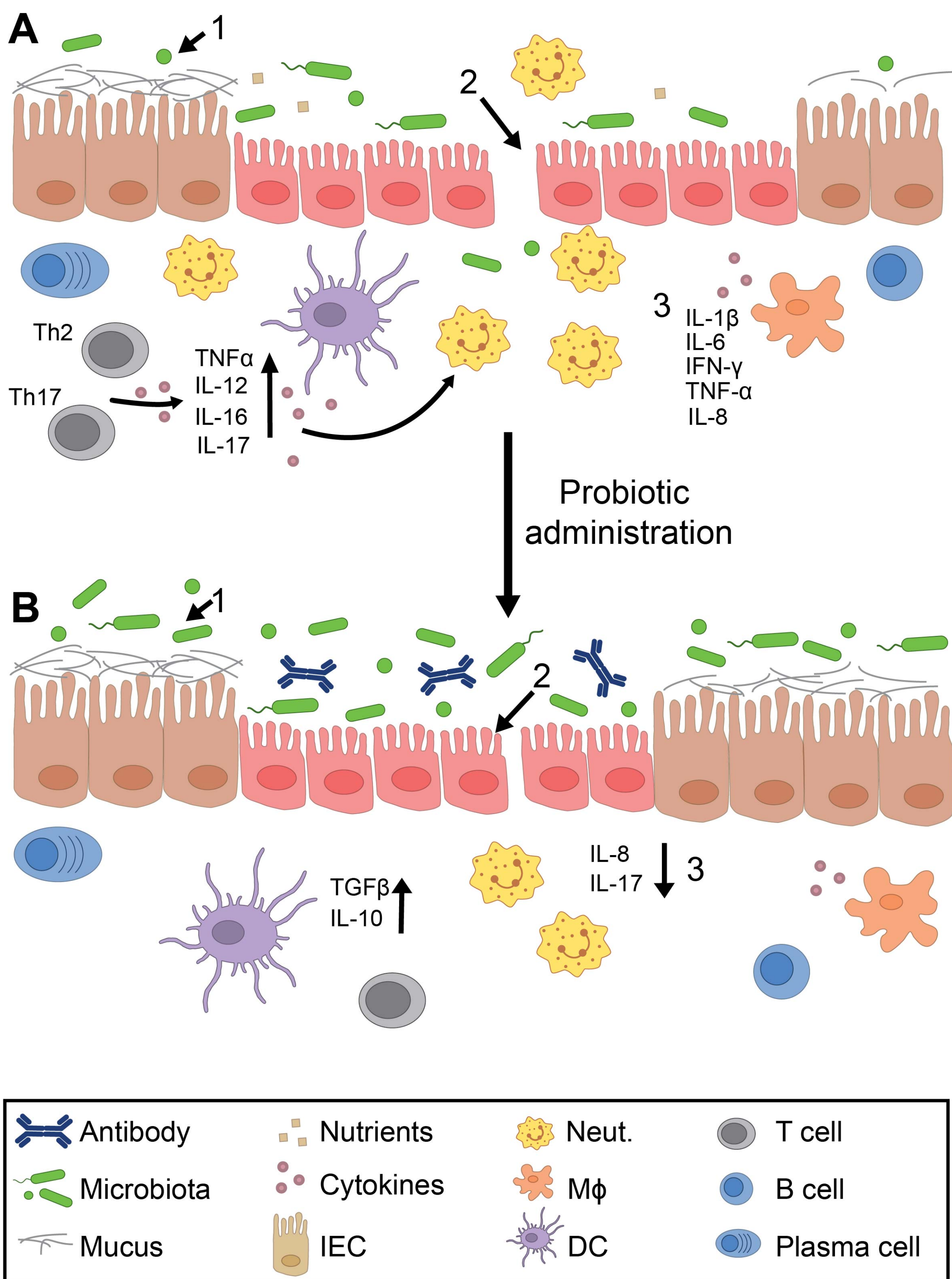
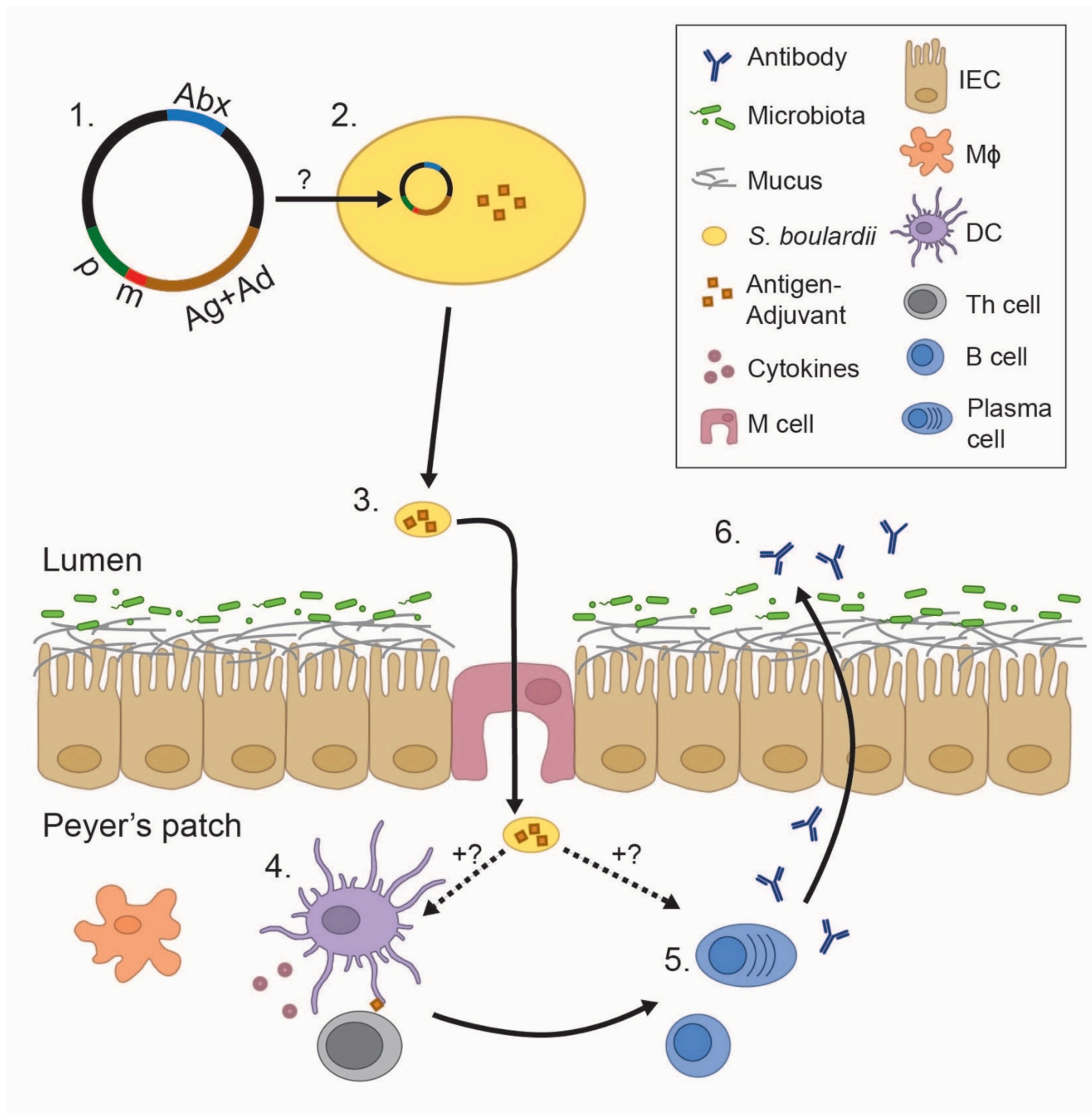


1442

1443

1444 **Fig 4.5 Potential effects of probiotics in UC**

1445 (A) Ulcerative colitis is characterized by (1) an altered microbiota of decreased diversity,
1446 (2) damage to the gastrointestinal epithelium, as well as (3) aberrant, overly inflammatory
1447 host immune responses. (B) By helping to (1) maintain a normal microbiota and (2)
1448 reinforce barrier function of the epithelium, probiotics may limit exposure to
1449 inflammatory signals. (3) Modulation of the mucosal immune system, including the
1450 cytokine milieu, neutrophil infiltration and function, and T cell differentiation, may also
1451 help redress aberrant responses to luminal antigens and prevent host-mediated damage to
1452 the mucosa. Abbreviations: IEC (intestinal epithelial cell), Neut. (neutrophil), Mϕ
1453 (macrophage), DC (dendritic cell).
1454



1455

1456 **Fig 4.6 Proposed model for development of an *S. boulardii*-based oral vaccine**

1457 **delivery system**

1458 (1) Plasmid design for heterologous protein expression in WT *S. boulardii* will require

1459 cloning of antibiotic resistance markers (Abx) and coding sequences for model vaccine

1460 antigens and adjuvants (Ag + Ad). Myc tags (m) will enable efficient identification of

1461 proteins expressed. (2) Optimization of transformation protocols (?) will be required to

1462 efficiently generate *S. boulardii* cells that express cytosolic vaccine antigens and

1463 adjuvants. (3) Recombinant yeast will protect vaccine antigens and adjuvants from
1464 degradation within the intestinal lumen and facilitate uptake through Peyer's patch M
1465 cells. (4) *S. boulardii* may have immunomodulatory effects on multiple cell types with
1466 the potential to affect vaccine responses (+?). DC uptake of yeast expressing both antigen
1467 and adjuvant will stimulate activation, inflammatory cytokine secretion, and presentation
1468 of antigen to T helper cells. (5) B cell activation and affinity maturation will lead to
1469 production of high affinity antibodies against vaccine antigen that (6) are secreted into
1470 the lumen to mediate protection against challenge. Abbreviations: p, promoter; m, myc
1471 tag; Ag, vaccine antigen; Ad, vaccine adjuvant; Abx, antibiotic selection marker; DC,
1472 dendritic cell; IEC, intestinal epithelial cell; Mϕ, macrophage.
1473
1474

1475 **Table 4.1 Clinical Trials Evaluating Probiotic Efficacy in Preventing Primary and Recurrent CDI**

Name	Year	Species (daily dose)	Endpoint	Patient population	Conclusions
Bacteria Trials Showing Benefit					
Hickson et al. [354]	2007	*Lactobacillus casei, Lactobacillus bulgaricus, Streptococcus thermophilus* ($4.2x10^{10}$ CFU)	Primary CDI	112 adults	Decreased incidence of primary CDI in patients receiving antibiotics when given probiotic bacteria
Gao et al. [355]	2010	*Lactobacillus acidophilus* CL1285, *L. casei* LBC80R ($5x10^{10}$ CFU or 10^{11} CFU)	Primary CDI	255 adult inpatients	Low and high dose probiotic mixtures confer protection against acquisition of primary CDI in adult patients
Bacteria Trials Showing No Benefit					
Thomas et al.[356]	2001	*Lactobacillus rhamnosus* GG (LGG) ($2x10^{10}$ CFU)	Primary CDI	267 adults	No statistically significant difference in primary CDI in adults with probiotic administration
Plummer et al. [357]	2004	*L. acidophilus, Bifidobacterium bifidum* ($2x10^{10}$ CFU)	Primary CDI	138 adults over 65 years old	No statistically significant difference in primary CDI in elderly patients with probiotic administration
Lawrence et al. [358]	2005	LGG (80mg lyophilized LGG given with 640 mg inulin)	Recurrent CDI	15 adults with recurrent CDI	No significant difference in recurrent CDI detected
Stein et al. [359]	2007	*L. acidophilus, B. bifidum, L. bulgaricus, S. thermophilus* ($1.5x10^{9}$ CFU of each)	Primary CDI	42 adults	No statistically significant difference in primary CDI in adults with probiotic administration

Beausoleil et al. [360]	2007	*L. acidophilus* CL1285, *L. casei* LBC80R ($5x10^{10}$ CFU)	Primary CDI	89 adult inpatients	No statistically significant difference in primary CDI with probiotic administration
Safdar et al. [361]	2008	*L. acidophilus* (Florajen) ($6x10^{10}$ CFU)	Primary CDI	40 adult inpatients	No statistically significant difference
Wullt et al. [362]	2009	*L. plantarum* 299v ($5x10^{10}$ CFU)	Recurrent CDI	20 adults with at least one CDI episode in previous 2 months	No statistically significant difference
Sampalis et al. [363]	2010	BIO-K+CL128 (*L. acidophilus* CL1285 and *L. casei*) (49 g then 98 g)	Primary CDI	437 adult inpatients	No statistically significant difference
Allen et al. [91]	2013	*L. acidophilus* CUL60 and CUL21, *B. bifidum* CUL20 and CUL34 ($6x10^{10}$ CFU)	Primary CDI	2941 adult inpatients over 65 years old	No statistically significant difference in primary CDI with probiotic administration
S. boulardii **Trials Showing Benefit**					
McFarland et al. [364]	1994	*S. boulardii* (1 g; $3x10^{10}$ CFU)	Recurrent CDI	124 adults with initial and recurrent CDI	Combination of antibiotic and *S. boulardii* therapy decreases CDI recurrence relative to antibiotics alone
Surawicz et al. [365]	2000	*S. boulardii* (1 g)	Recurrent CDI	32 adults with CDI	Statistically significant decrease in CDI recurrence with *S. boulardii* administration in combination with high dose vancomycin, but not metronidazole or low dose vancomycin

Kotowska et al. [366]	2005	*S. boulardii* (500 mg)	Primary CDI	246 children treated for otitis media or respiratory infections	*S. boulardii* decreased the risk of CDI in children receiving antibiotics, although with a borderline level of significance
***S. boulardii* Trials Showing No Benefit**					
Surawicz et al. [367]	1989	*S. boulardii* (1 g)	Primary CDI	180 adult patients	No statistically significant decrease in CDI
Surawicz et al. [368]	1989	*S. boulardii* (1 g)	Recurrent CDI	13 patients	Non statistically significant decrease in CDI diarrhea with *S. boulardii* administration
McFarland et al. [369]	1995	*S. boulardii* (1 g; $3x10^{10}$ CFU)	Primary CDI	193 adult patients receiving antibiotics	No significant difference in incidence of primary CDI between groups
Lewis et al. [370]	1998	*S. boulardi*i (226 mg)	Primary CDI	69 patients over 65 years old receiving antibiotics	No statistically significant difference in incidence of CDI
Can et al. [371]	2006	*S. boulardi*i ($1x10^{10}$ CFU)	Primary CDI	151 adults receiving antibiotics	No statistically significant difference in incidence of CDI

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1477 **Table 4.2 Effects of Probiotics on the Gastrointestinal Epithelium**

Genus	Species	Strain or Company	Effects on Epithelial Barrier	Model System	Reference
Gram-Positive Bacteria					
Lactobacillus	*L. rhamnosus*	GG (ATCC 53103)	TER ↑	Alcoholic liver disease in male Sprague–Dawley rats	Forsyth et al. 2009 [372]
	L. rhamnosus	GG (ATCC 53103)	Prevented ↓ in ZO-1, claudin-1, symplekin, p130, and fordin	Chronic alcohol feeding in mice	Bull-Otterson et al. 2013 [273]
	L. rhamnosus	GG (ATCC 53103)	Occludin, claudin-1, ZO-1 ↑ when given with gliadin	Caco-2 cells	Orlando et al. 2014 [373]
	L. rhamnosus	GG (ATCC 53103) p40 and p75	PKCε and PKCβI membrane translocalization; prevent occludin, ZO-1, E-cadherin, and B-catenin redistribution in ERK1/2 and PKC dependent manners	Caco-2 cells exposed to H_2O_2	Seth et al. 2008 [374]
	L. rhamnosus	GG (ATCC 53103)	TER, claudin-1, ZO-1, and occludin ↑	*In vitro* human epidermal keratinocytes	Sultana et al. 2013 [168]
	L. rhamnosus	ATCC 7469	ZO-1, TLR2, and TLR4 ↑; PKCα unchanged; prevent mucus disruption	ETEC-infected IPEC-J2 cells	Zhang et al. 2015 [154]
	L. acidophilus	ATCC4356	TER ↑, ↑ occludin and ZO-1 phosphorylation	Control and EIEC-infected Caco-2 cells	Resta-Lenert & Barrett 2003 [375]
	L. plantarum	ATCC 10241	Transient TER ↑	*In vitro* human epidermal keratinocyte	Sultana et al. 2013 [168]
	L. plantarum	CGMCC 1258	Prevented ↓ in occludin	ETEC-infected piglets	Yang et al. 2014 [376]

	L. plantarum	299v	No change in bacterial translocation to cervical and mesenteric lymph nodes	5-FU treated rats	Von Bültzingslöwen et al. 2003 [377]
Streptococcus	*S. thermophilus*	*ATCC19258*	*TER* ↑, ↑ occludin and ZO-1 phosphorylation	Control and EIEC-infected Caco-2 cells	Resta-Lenert & Barrett 2003 [375]
Bifidobacterium	*B. bifidum*	WU12	↑ occludin mRNA in Caco-2 cells after TNF-α exposure	Caco-2 cells	Hsieh et al. 2015 [174]
	B. longum	ATCC 51870	TER (TLR2 dependent), claudin-1 and -4, ZO-1, and occludin ↑	*In vitro* human epidermal keratinocytes	Sultana et al. 2013 [168]
	B. infantis	isolated from VSL#3	Prevented TNF-a- and IFN-γ-induced TER ↓, ↑ claudin 3 and 4, occludin, and ZO-1; prevented redistribution of claudin-1 and occludin in vivo	T84 cells, IL-10 deficient mice	Ewaschuk et al. 2008 [378]
Gram-Negative Bacteria					
Escherichia	*E. coli*	Nissle 1917	↑ ZO-1 in absence of inflammation; ↑ ZO-1 and ZO-2 in DSS colitis; ↓ recruitment of inflammatory leukocytes to colon	Monoassociated mice and DSS colitis	Ukena et al. 2007 [379]
Probiotic Cocktails					
	L. rhamnosus and *L. helveticus*	R0011 and R0052 (Lacidofil)	Intestinal permeability ↓, ↓ bacterial adherence to epithelium	Chronic stress in rats	Zareie et al. 2006 [380]
	S. thermophilus and *L. acidophilus*	ATCC19258 and ATCC4356	↑ TER, ↑ phosphorylation of occludin and ZO-1	Caco-2 cells, EIEC infected Caco-2 cells	Resta-Lenert & Barrett 2003 [375]
Yeast					

Saccharomyces	*S. boulardii*	Biocodex	No change in TER in T84 control or infected cells; partial protection from ↑ HRP flux in *Shigella flexneri* coinfection; restoration or preservation of claudin-1 and ZO-2 expression in later time points	T84 control and *Shigella flexneri* infected cells	Mumy et al. 2008 [173]
	S. boulardii	Biocodex	Prevents EPEC-induced activation of the ERK1/2 mitogen-activated protein (MAP) kinase pathway; preservation of ZO-1 distribution	EPEC stimulated T84 cells	Czerucka et al. 2000 [169]
	S. boulardii	Biocodex	Prevented EHEC-induced MLC phosphorylation linked to ↓ TER	EHEC infected T84 cells	Dahan et al. 2003 [329]
	S. boulardii	Biocodex	Inhibited IL-1β and TcdA-induced ↑ in IL-8 expression, Erk1/2 and JNK/SAPK but not p38 activation; ↓ ERK1/2 activation in TcdA-treated ileal loop	NCM460 human colonocytes; mouse ileal loop	Chen et al. 2006 [381]
	S. boulardii	Perenterol	↑ brush border enzyme activity	Duodenal biopsies of *S. boulardii*-treated healthy human volunteers	Jahn et al. 1996 [382]
	S. cerevisiae	CNCM I-3856	No effect on barrier function	IPEC-1 cells with ETEC exposure	Zanello et al. 2011 [157]

1478 Table 4.2 abbreviations: DSS (dextran sodium sulfate); EHEC (enterohemorrhagic *E. coli*); EIEC (enteroinvasive *E. coli)*; EPEC
1479 (enteropathogenic *E. coli*); Erk1/2 (extracellular signal–regulated kinases 1/2); HRP (horseradish peroxidase); IPEC-1 (newborn piglet
1480 intestinal epithelial cell line); JNK/SAPK (c-Jun amino-terminal kinase/stress-activated protein kinase); MLC (myosin light chain);
1481 PKC (protein kinase C); TER (transepithelial resistance).
1482

1483 **Table 4.3 Clinical Trials Evaluating Probiotic Efficacy in Maintenance or Induction of UC Remission**

Name	Year	Species (daily dose)	Primary Outcomes	Patient population	Conclusions
Probiotic Trials Showing Benefit					
Zocco et al. [383]	2006	LGG (1.8 x 10^{10} CFU with or without mesalazine)	Relapse (UC symptoms requiring treatment, or increase in CAI to > 4)	187 patients with UC in remission < 12 months	Increased relapse-free time with probiotics relative to mesalazine, no difference in relapse rate at 6 or 12 months
Furrie et al. [384]	2005	*Bifidobacterium. longum* (2x10^{11} CFU) plus Synergy (6g fructooligosaccharide/ inulin) twice daily	CAI, bowel habit index, sigmoidoscopy score, histology score, and immune parameters (colonic TNF-α, IL-1α, serum C reactive protein, human beta defensins)	16 patients with active UC	CAI significantly reduced in probiotic group, TNF-α and IL-1α lower in the probiotic compared to the placebo group after 4 weeks (P = 0.0177 and P = 0.0051 respectively), defensin levels not different
Kruis et al. [385]	1997	*E. coli* Nissle 1917 (2.5x10^{10} viable CFU daily for four days and twice daily for the remainder of the study)	Time to relapse (CAI ≥ 4)	120 patients with chronic UC in remission	*E. coli* (16% relapse rate) as effective as mesalazine-treated (11.3% relapse rate) in maintaining remission
Rembacken et al. [306]	1999	*E. coli* Nissle 1917 (2 capsules with 2.5 x10^{10} CFU viable bacteria twice a day)	Time to remission, rate of relapse after induction of remission	116 patients with clinically active UC	*E. coli* Nissle 1917 plus steroids similar to mesalazine plus steroids to induce remission (OR 1.35 (95%CI 0.6 to 3.04), relapse rate lower in the probiotic group (67% v. 73% in controls, $p < 0.05$), no difference in duration or mean time to remission

Kruis et al. [14]	2004	*E. coli* Nissle 1917 (2.5-25x10^9 viable CFU once daily for four days, twice daily for the remainder of study)	Time to relapse (CAI >6 or increase of 3 points and CAI > 4; endoscopic index > 4 and histological signs of acute inflammation)	327 patients with UC in remission	*E. coli* Nissle 1917 (36.4% relapse) as effective as mesalazine (33.9% relapse) in maintaining remission
Matthes et al. [305]	2010	*E. coli* Nissle 1917 (daily 40, 20, or 10 ml enemas containing 10^8 CFU/ml)	Clinical remission (DAI ≤ 2)	90 patients with moderate distal activity in UC	Dose dependent increase in remission with *E. coli* therapy (by per protocol but not intention to treat analysis), time to remission smallest with highest dose
Bibiloni et al. [386]	2005	VSL#3 (1.8x10^{12} CFU twice daily)	Remission (UCDAI ≤ 2) or response (UCDAI decrease ≥ 3 pts)	Adult patients with ≥ 2 week history of active UC not responsive to mesalamine	VSL#3 promotes remission
Miele et al. [15]	2009	VSL#3 (weight-based dose between 4.5-18 x10^{12} CFU)	Remission rate and time to relapse	29 pediatric patients with newly diagnosed UC	VSL#3-treated patients more likely to achieve remission, had fewer relapses and lower endoscopic and histological scores at 6 and 12 months or point of relapse
Tursi et al. [387]	2010	VSL#3 (3.6x10^{12} viable lyophilized bacteria)	Decrease in UCDAI of ≥ 50%	144 patients with mild to moderate relapsing UC	VSL#3 reduced UCDAI scores (3 points or more) and rectal bleeding, but not endoscopic scores or physician's rate of disease activity, trend toward increased remission in the VSL#3 group (P = 0.069)

Sood et al. [388]	2009	VSL#3 ($3.6x10^{12}$ lyophilized bacteria)	50% reduction in UCDAI score	147 adult patients with mild-to-moderate UC	VSL#3 significantly better than placebo in inducing remission and improving UCDAI scores (p<.001)
Venturi et al. [389]	1999	VSL#3 (3 g twice daily for 12 months)	Remission maintenance	20 patients with UC intolerant to 5-ASA in remission	15/20 VSL#3 treated patients (75%) still in remission at 12 months
Li et al. [390]	2012	Bifid Triple Viable (6 capsules of *Bacillus acidophilus*, *B. bifidum*, and *Streptococci*)	Clinic symptom score, colon mucosa inflammation score, and immune indices	82 adult patients with active UC	Decreased clinical symptoms and mucosal inflammation scores in probiotic group
Tsuda et al. [267]	2007	BIO-THREE (2mg *Enterococcus faecalis* T-110, 10 mg *Clostridium butyricum* TO-A and 10 mg *Bacillus mesentericus* TO-A)	Improved UCDAI scores	20 patients with mild to moderate UC	Remission (UCDAI score < 2) in 45% of patients (9/20) and response (decrease in UCDAI > 3) in 10% of patients (2/20)
Ishikawa et al. [391]	2003	Yakult (*B. breve, B. bifidum, L. acidophillus* YIT 0168 in 100mL with at least 10^{10} viable bacteria)	Exacerbation of clinical symptoms (increased frequency of bowel movements or abdominal pain, or appearance or increased frequency of blood or mucus movements)	21 UC adult patients	Some protection in preventing exacerbation of UC symptoms (3/11 probiotic-treated versus 9/10 control patients developed exacerbated symptoms)
Guslandii et al. [392]	2010	*S. boulardii* (250 mg three times daily for 4 weeks)	Improved clinical score	25 patients with mild to moderate UC	Reduced CAI scores with *S. boulardii*
Probiotic Trials Showing No Benefit					

Tamaki et al. [393]	2015	*B. longum* 536 (2 doses $3x10^{11}$ freeze dried viable CFU three times daily)	Remission (UCDAI ≤ 2)	56 patients with mild to moderate UC	No difference in remission or UCDAI scores in probiotic versus placebo (containing dextrin) groups rectal bleeding reduced with probiotics
Tursi et al. [394]	2004	VSL#3 ($9x10^{11}$ lyophilized bacteria plus either balsalazide or mesalazine)	Remission (based on clinical evaluation and diary card)	90 patients with mild to moderate ulcerative colitis	Remission similar with VSL#3 plus balsalazide and placebo plus balsalazide, but time to remission shorter in probiotic group (4 v. 7 average days in probiotic and placebo groups, p <0.01)
Ng et al. [395]	2010	VSL#3 ($3.6x10^{12}$ bacteria)	Clinical response and remission as defined by UCDAI	28 patients with mild to moderate UC	10/14 VSL#3 treated patients showed a clinical response relative to 5/14 in control patients (P = 0.064)
Kato et al. [396]	2004	*B. breve, B. bifidis*, and *L. acidophilus* ($1x10^{10}$ CFU in fermented milk plus sulfasalazine or 5-ASA)	Remission rate and CAI	20 patients with moderate to severe UC	No improvement in CAI scores over placebo (OR 0.64 (95% CI 0.10 to 4.10)), improved endoscopic activity index score (p < 0.01) and histological scores (p < 0.01) in probiotic group versus no improvement in placebo group
Wildt et al. [397]	2011	Probio-Tec AB-25 (1.25 x 10^{10} CFU of both *L. acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12)	Time to relapse (SCCAI >4 or endoscopic changes)	32 patients in remission ≥ 4 wks	No significant clinical benefit for maintaining remission

1484 Table 4.3 abbreviations: CAI (clinical activity index); DAI (disease activity index); UCDAI (ulcerative colitis daily activity index);
1485 SCCAI (simple clinical colitis activity index); 5-ASA (5-aminosalicylic acid). Scoring criteria for CAI, DAI, UCDAI, and SCCAI
1486 differ between studies.
1487

1488 **Table 4.4 Immunological Effects of Probiotic Strains**

Genus	Species	Strain	Effects on Immune System	Model	Reference
Gram-Positive Bacteria					
Lactobacillus	*L. acidophilus*	NCFMTM	Induced IL-12p70 and IL-10 in a dose dependent manner; ↑ IL-23, IL-6, IL-12p40, and CXCL1 expression	Human monocyte derived DCs	Gad et al. 2011 [283]
	L. acidophilus	X37	Strong ↑ IL-12 and TNF-α; ↑ activation markers CD40, CD83, CD86, HLA-DR	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
	L. fermentum	CECT 5716	↓ IL-6 at week 2 in therapeutic group relative to TNBS only controls; no effect on MPO levels	TNBS colitis	Mane et al. 2009 [301]
			↓ colonic MPO levels relative to TNBS controls, ↓ TNF-α relative to controls	TNBS colitis	Peran et al. 2007 [398]
	L. fermentum	Lf1	↑ *SOD1* expression	DSS colitis	Chauhan et al. 2014 [399]
	L. paracasei	Z11	Strong ↑ IL-12 and TNF-α; ↑ activation markers CD40, CD83, CD86, HLA-DR	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
	L. plantarum	HY115	↓ IL-1β, IFN-γ, TNF-α expression compared to DSS controls	DSS colitis	Lee et al. 2008 [303]
			↑ cytoplasmic IkBa and decreased nuclear NF-κB compared to DSS controls		
	L. brevis	HY7401	↓ IL-1β, IFN-γ, TNF-α expression compared to DSS controls; decreased intestinal epithelial MPO activity compared to DSS-treated; ↑ cytoplasmic IkBa and ↓ nuclear NF-κB compared to DSS controls	DSS colitis	Lee et al. 2008 [303]

	L. reuteri	ATCC55730	↓ TNF-α relative to TNBS controls	TNBS colitis	Peran et al. 2007 [398]
	L. reuteri	R2LC, JCM 5869, ATC PTA 4659, ATCC 55730	Prevented ↑ in colonic P-selectin, ↓ numbers of rolling leukocytes in submucosal and mucosal vessels	DSS colitis	Schreiber et al. 2009 [400]
	L. reuteri	DSM 12246:12002	Strong ↑ IL-10	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
	L. salivarius	Ls-33	↑ IL-10 secretion, IL-12p40, IL-23, IL-6, and CXCL1 expression	Human monocyte derived DCs	Gad et al. 2011 [283]
	L. rhamnosus	GG (ATCC 53103)	↓ IL-23, IL-17, and CD40 expression	LPS stimulated T84 and HT29 3D cultures	Ghadimi et al. 2012 [297]
	L. rhamnosus	GG (ATCC 531030)	↓ hepatic MPO levels (neutrophil infiltration)	Alcohol-fed rats	Forsyth et al. 2009 [372]
	L. rhamnosus	GG (ATCC 53103)	Prevented ↑ in hepatic TNFα levels	Chronic alcohol feeding in mice	Bull-Otterson et al. 2013 [273]
	L. rhamnosus	ATCC 7469	↑ TLR2 and TLR4 expression; ↓ TNF-α with pretreatment	ETEC infeted IPEC-J2 cells	Zhang et al. 2015
Bifidobacterium	*B. infantis*	35624	↑ IL-10 secretion; ↓ IL-12p70 secretion and ↑ IL-10 secretion in LPS-, IFNγ-, and TNFα-stimulated cells; ↑ IL-23, IL-12p40, IL-6, and CXCL1 expression	Human monocyte derived DCs	Gad et al. 2011 [283]

B. infantis	Guangzhou Baoxing Biotechnology Company	↑ IL-2, IL-12p40, RORγT, IL-23, IL17A expression in MLNs relative to untreated TNBS controls	TNBS colitis	Zuo et al. 2014 [401]
B. infantis	Riken lab	↓ IL-17 production in *ex vivo* stimulated colonocytes	DSS-stimulated mouse colonocytes	Tanabe et al. 2008 [284]
B. infantis	JCM 1222	↓ IL-17A and IFN-γ, and ↑ IL-10 expression in T cells; ↓ CD40 and CD80 expression on IECs	T cells stimulated *ex vivo* with IECs from DSS-treated mice; DSS-treated mice	Miyauchi et al. 2013 [298]
B. infantis	isolated from VSL#3	↓ IFN-γ secretion, ↑ TGFβ	IL-10 deficient mice	Ewaschuk et al. 2008 [378]
B. animalis subsp. lactis	Bb12	Strong ↑ IL-10	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
B. bifidum	Riken lab	↓ IL-17 production in *ex vivo* stimulated colonocytes	DSS-stimulated mouse colonocytes	Tanabe et al. 2008 [284]
B. bifidum	S131, Z9	Strong ↑ IL-10	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
B. catulenatum	Riken lab	↓ IL-17 production in *ex vivo* stimulated colonocytes	DSS-stimulated mouse colonocytes	Tanabe et al. 2008 [284]
B. breve	DSMZ 20213	↓ IL-23, IL-17, and CD40 expression	LPS-stimulated T84 and HT29 3D cultures	Ghadimi et al. 2012 [297]

	B. longum	Q45, Q46	Strong ↑ IL-10	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
Gram Negative Bacteria					
Escherichia	*E. coli*	Nissle 1917	↑ IL-12p70 and IL-10 in a dose dependent manner; ↓ IL-12p70 secretion and ↑ IL-10 secretion in LPS, IFN-γ, and TNF-α stimulated moDCs; ↑ IL-23 and IL-6 expression; no change in IL-17	Human monocyte derived DCs	Gad et al. 2011 [283]
	E. coli	Nissle 1917 O6:K5:H1; F18 OR:K1:H5 BJ4 OR:K:H2; MG1655 OR:K:H48; UTI	Strong ↑ IL-10; weak ↑ TNF-α and IL-12p70	Human monocyte derived DCs	Zeuthen et al. 2006 [288]
Probiotic cocktails					
Duolac Gold (mixture of 6 probiotics)	*B. lactis, B. longum, B. bifidum, L. acidophilus, L. rhamnosus, Streptococcus thermophilus*	KCTC 11904BP, 12200BP, 12199BP; KCTC 11906BP, 12202BP; KCTC 11870BP	↓ IL-6 in serum and colon relative to DSS control mice	DSS colitis	Yoon et al. 2014 [302]
VSL#3	*L. casei, L. plantarum, L. acidophilus, L.*	VSL pharma-ceuticals	↑ primarily IL-12p70 over IL-10; ↑ IL-23, IL-12p40, IL-6, and CXCL1 expression	Human monocyte derived DCs	Gad et al. 2011 [283]

VSL#3	*delbrueckii subsp. bulgaricus, B. longum, B. breve, B. infantis*, and *Streptococcus salivarius* subsp. *thermophilus*		↑ IL-10 and ↓ IL-12 in colonic lamina propria DCs; no change in IL-6 or IL-13	Patients with mild to moderate UC	Ng et al. 2010 [395]
Mix 1	*Lactobacillus acidophilus* Bar 13 and *Bifidobacterium longum* Bar 33 (1:1)	Barilla G&R f.lli SPA (Parma, Italy)	↓ serum IL-12, TNF-α, MCP-1, and IFN-γ and ↑ IL-10 compared to TNBS -treated; ↓ total $CD4^+$ cells and ↓ $γδ^+$ lamina propria T cells, ↑ Tregs relative to TNBS-treated controls;	TNBS colitis in mice	Roselli et al. 2009 [277]
Mix 2	*L. plantarum* Bar 10, *Streptococcus thermophilus* Bar 20, and *B. animalis subsp. lactis* Bar 30 (1:1:1)	Barilla G&R f.lli SPA (Parma, Italy)	↓ serum IL-12, TNF-α, MCP-1, and IFN-γ and ↑ IL-10 compared to TNBS -treated	TNBS colitis in mice	Roselli et al. 2009 [277]
Yeast					
Saccharomyces	*S. boulardii*	Biocodex	↓ plasma IL-6, IL-4, IL-1β, and TNF-α compared to control mice	Db/db mice	Everard et al. 2014 [274]
	S. boulardii	Biocodex	Prevented IL-8 ↑ (viable but not heat killed *S. boulardii*); viable yeast prevented EHEC induced NFκB DNA binding and MAPK activation	EHEC infected T84 cells	Dahan et al. 2003 [329]

S. boulardii	Biocodex	*S. boulardii* supernatant ↓ IL-8 mRNA and protein production, prevented IkBa degradation, and reduced NFκB DNA binding	IL-1β and TNFα stimulated HT-29 cells	Sougioultzis et al. 2006 [294]
S. boulardii	Biocodex	↓ ERK, JNK, and NFκB activation and IL-8 secretion	*S. flexneri* stimulated T84 cells	Mumy et al. 2008 [173]
S. boulardii	Floratil	↑ serum IL-10 (viable or heat killed *S. boulardii*)	Murine intestinal obstruction model	Generoso et al. 2011 [138]
S. boulardii	Bioflor	↑ PPARγ expression; ↓ IL-8 secretion from unstimulated and stimulated HT-29 cells in a PPARγ dependent manner; ↑ PPARγ ↓ IL-8, IL-1β, IL-6, IL-8R, TNF-α , and iNOS expression in healthy and TNBS treated colon	TNF-α- and IL-1β-stimulated HT-29 cells; TNBS colitis in rats	Lee et al. 2009 [402]
S. boulardii	Merck SA	↑ Kupffer cells; earlier and higher levels of TNF-α , IL-12, and IFN-γ in response to *E. coli* B41 infection	Monoassociated Swiss/NIH mice	Rodrigues et al. 2000 [137]
S. boulardii	Biocodex	↓ T cell infiltrate and NF-κB activation in colon; no ↑ in IL-10, TGFβ in MLN or colon; no ↑ in CXCL9, CXCL10, CXCL11, CCL4, CCL5 in MLNs; ↑ P-selectin interaction with activated T cells and ↑ T cell accumulation in MLNs	Lymphocyte transfer SCID mouse model of colitis	Dalmasso, Cottrez et al. 2006 [403]
S. boulardii	Biocodex	Preincubation prevents TNF-α expression and caspase 8 and 9 activation, ↓ IL-8, IL-6, IL-1β, TNF-α and IFN-γ	T84 human colonic cell line	Dalmasso, Loubat, et al. 2006 [404]

S. boulardii	Perenterol	No effect on sIgA; ↑ CD25 expression by peripheral CD4+ T cells; no change in peripheral B or T cell numbers	PMBCs of *S. boulardii*-treated healthy human volunteers	Jahn et al. 1996 [382]
S. boulardii	Ultra-Levure, Biocodex	Reduced iNOS in macrophages; high dose *S. boulardi*i reduced colon citrulline in diarrhea	IFNγ stimulated mouse macrophage cells RAW 264-7; castor oil induced diarrhea in male Wistar rats	Girard et al. 2005 [405]
S. boulardii	Biocodex	↓ ERK, JNK, and NFκB activation; IL-8 secretion from control and infected cells; ↓ PMN transmigration across infected T84 cells or recruitment to human fetal colonic xenografts	*Shigella flexneri* infected T84 cells and human fetal colonic xenografts	Mumy et al. 2008 [173]
S. boulardii	Biocodex	*S. boulardii* and supernatant ↓ CD40, CD80, CCR7, TNF-α, and IL-6 expression; *S. boulardii* ↑ IL-10 expression	LPS-stimulated human myeloid CD1c+CD11c+CD123–DCs	Thomas et al. 2009 [290]
S. boulardii	Reflor, Biocodex	↓ serum NO production compared to TNBS controls	TNBS colitis in rat	Soyturk et al. 2012 [406]
S. boulardii	Ardey-pharm	Small ↑ TNF-α and CXCL1 expression; little effect on IL-10, IL-12p70	Human monocyte derived DCs	Gad et al. 2011 [283]
S. boulardii	Biocodex	↑ ileal expression of chemokine KC	TcdA-treated mouse ileal loop	Chen et al. 2006 [381]

S. boulardii	Biocodex	No change in total lymphocyte number or serum antibody levels; cell wall binds complement C3b; ↑ leukocyte chemokinesis; ↑ erythrocyte, total leukocyte, neutrophil, and polynuclear cell numbers	Healthy human PBMCs	Caetano et al. 1986 [407]
S. cerervisiae	CNCM I-3856	↓ IL-6 and IL-8 secretions and CCL20, CXCL2 and CXCL10 expression	ETEC stimulated porcine epithelial IPEC-1 cells	Zanello et al. 2009 [157]

1489 Table 4.4 abbreviations: CXCL (C-X-C motif ligand chemokine); DSS (dextran sodium sulfate); HLA-DR (human leukocyte antigen -
1490 antigen D related); iNOS (inducible nitric oxide synthase); MPO (myeloperoxidase); NF-κB (nuclear factor kappa-light-chain-
1491 enhancer of activated B cells); PBMC (peripheral blood mononuclear cell); PPARy (peroxisome proliferator-activated receptor y);
1492 RORγT (retinoic acid receptor-related orphan receptor gamma t); SCID (severe combined immunodeficiency); sIgA (secretory
1493 immunoglobulin A); SOD1 (superoxide dismutase 1); TGFβ (transforming growth factor β); TNBS (2,4,6-trinitrobenzenesulfonic
1494 acid).

5) General Materials and Methods

The following is adapted from the article by Lauren E. Hudson, Taryn P. Stewart, Milo B. Fasken, Anita H. Corbett, and Tracey J. Lamb entitled "Transformation of Probiotic Yeast and their Recovery from Gastrointestinal Immune Tissues Following Oral Gavage in Mice" published in 2016 in the *Journal of Visualized Experiments* (doi: 10.3791/53453).

Probiotic microorganisms are an intriguing potential means of efficiently and economically delivering heterologous proteins to the gastrointestinal tract. These organisms are capable of surviving passage through the gastrointestinal tract yet do not colonize it[309], enabling controlled dosing and limiting exposure to the drug expressed. Furthermore, the ability to easily engineer these organisms to produce heterologous protein on a large scale renders them an economical alternative to synthetic delivery particles. However, development of such an approach, as recently demonstrated using an auxotrophic strain of the probiotic yeast *Saccharomyces boulardii*[408], requires knowledge of laboratory techniques not traditionally combined within a given study, ranging from yeast and molecular biology to animal handling techniques and immunological methods. Thus although the individual procedures described herein are not in themselves novel laboratory protocols, the goal of this manuscript is to present a unified introduction to techniques needed for experimental testing of probiotic yeast as drug delivery vehicles to the murine gastrointestinal tract. Provided is a compilation of essential protocols for: 1) generation of auxotrophic mutant strains of yeast that can easily be genetically manipulated; 2) transformation of yeast cultures to express heterologous protein; 3)

1519 administration of recombinant yeast to the intestine via oral gavage; and 4) recovery of
1520 viable recombinant probiotic yeast from the murine intestine and assessment of their
1521 heterologous protein expression.

1522 First, although numerous positive and negative selection methods exist for the
1523 manipulation of yeast species, negative selection such as through the use of auxotrophic
1524 markers increases both the efficiency and ease with which yeast can be transformed and
1525 selected. Positive selection of transformants using antibiotics, in contrast, significantly
1526 increases the cost of yeast manipulation. Furthermore, selection of yeast on antibiotic-
1527 containing solid media can allow for increased growth of untransformed background
1528 colonies relative to selection of auxotrophic yeast on synthetic drop out solid media
1529 (unpublished observations). Auxotrophic yeast are strains which lack enzymes critical for
1530 the synthesis of essential amino acids or uracil. Such yeast can grow only if
1531 supplemented with the missing metabolite or metabolic gene, thus enabling negative
1532 selection when yeast are plated onto synthetic drop out media that lacks the essential
1533 metabolite. Many commonly used *Saccharomyces cerevisiae* laboratory strains are in fact
1534 already auxotrophic mutants[409]. Industrial, clinical, and probiotic yeast strains, however,
1535 are typically prototrophic with the ability to synthesize all required nutrients. To enable
1536 more efficient genetic manipulation of such yeast, auxotrophic genes can be selectively
1537 targeted to generate strains that can be selected without antibiotics. Specific targeting of
1538 auxotrophic marker genes can be achieved through PCR-mediated gene disruption
1539 relying on homologous recombination or more recently through CRISPR/Cas9
1540 targeting[410–412]. Alternatively, UV mutagenesis can quickly generate auxotrophic mutants
1541 even in yeast strains for which transformation with multiple plasmids is technically

1542 difficult[332]. While PCR targeting and CRISPR/Cas9 have been described extensively
1543 elsewhere, presented in part one of this manuscript is a detailed protocol describing a UV
1544 mutagenesis approach to create auxotrophic strains that will allow for negative selection
1545 rather than positive antibiotic selection of yeast transformants.

1546 The next necessary step in the use of such auxotrophic strains for oral delivery of
1547 heterologous protein is yeast transformation with plasmid DNA. Since the first successful
1548 transformation of yeast spheroplasts reported for *Saccharomyces cerevisiae* in 1978[413],
1549 numerous modifications have been characterized to increase the efficiency and ease with
1550 which yeast species can be genetically modified. Use of electroporation for the successful
1551 transformation of DNA into *S. cerevisiae* was first described in 1985[414] and has since
1552 been improved via the addition of 1 M sorbitol incubation to osmotically support cells[415].
1553 Electroporation efficiency has furthermore been shown to depend on the yeast species
1554 and strain, cell number and phase of growth, electroporation volume, field strength, and
1555 specific buffers[416]. Lithium acetate (LiOAc) transformation, originally described by Ito *et*
1556 *al.*[417], is among the most commonly used transformation protocols as it requires no
1557 special equipment. Additional analyses showed that the efficiency of LiOAc yeast
1558 transformation greatly increases when cells are collected in mid-log phase of growth and
1559 are heat shocked in the presence of polyethylene glycol (PEG) and DNA at 42 °C[417].
1560 Incubation of whole intact yeast with PEG is essential for efficient transformation,
1561 possibly through improving attachment of DNA to the cell membrane as well as via other
1562 effects on the membrane[418]. Lithium itself also increases the permeability of intact
1563 cells[419]. Although most laboratory *S. cerevisiae* strains can easily be transformed using
1564 LiOAc transformation[409], other yeast species may be more efficiently transformed using

1565 alternative protocols. *Pichia pastoris*, for example, is most efficiently transformed via
1566 electroporation rather than LiOAc transformation[418]. It is crucial, therefore, to test
1567 multiple methods of transformation and to optimize incubation periods and reagent
1568 concentrations when attempting to genetically modify an uncharacterized yeast strain.
1569 This manuscript thus describes both LiOAc transformation and electroporation as
1570 techniques for the transformation of auxotrophic mutant and wild type *S. boulardii*.
1571 Interested readers are directed to recent reviews for thorough descriptions of the
1572 evolution of yeast transformation, alternative protocols, and further discussions of
1573 possible mechanisms of action[418,420]. Transformation of yeast with plasmid encoding an
1574 easily detectable protein is furthermore essential for downstream testing in order to
1575 ensure proper expression and function of heterologous protein. Myriad different proteins
1576 may be selected depending on the ultimate purpose of the therapeutic study and the
1577 antibodies available for protein detection by immunoblotting, ELISA, and other
1578 techniques. Protocols for these techniques have been thoroughly described
1579 elsewhere[421,422], and can be used to determine levels of heterologous protein production
1580 from transformed yeast by comparison to standard curves. For purposes of demonstration
1581 and to show successful production of a very commonly used protein in yeast biology, this
1582 manuscript presents transformation with plasmid encoding green fluorescent protein
1583 (GFP), which allows for subsequent detection using fluorescence microscopy.

1584 Equally important to the production of probiotic organisms that express
1585 heterologous protein is the proper administration and detection of these microorganisms
1586 within gastrointestinal tissues, as described in parts three and four. Administration of
1587 recombinant yeast via oral gavage allows for delivery of controlled quantities of yeast

1588 directly into the stomach, from which C57BL/6 mice are naturally incapable of
1589 vomiting[423]. However, improper animal handling and gavage can lead to esophageal
1590 damage and perforation, gastric perforation, tracheal administration, and aspiration
1591 pneumonia[424,425]. Poor technique and inexperience can furthermore increase variability in
1592 murine immune responses and experimental results, which have been attributed to animal
1593 stress upon oral gavage[426,427]. Practice in the proper technique can thus not only attenuate
1594 animal discomfort, but can also increase precision of experimental results. This
1595 manuscript describes and demonstrates animal handling and oral gavage for the
1596 administration of controlled doses of recombinant yeast.

1597 Finally, it is vital to confirm successful delivery of recombinant yeast by
1598 analyzing lymphoid tissues for the presence of yeast and heterologous protein. The
1599 gastrointestinal immune tissues which can most easily and predictably be examined for
1600 the presence of yeast are the Peyer's patches. Peyer's patches are secondary lymphoid
1601 organs along the small intestine that are key sites of mucosal immune response
1602 induction[428]. Antigens from the lumen are transferred transcellularly through microfold
1603 (M) cells in the epithelium and are released into the Peyer's patches, thus exposing
1604 enclosed antigen presenting cells to intestinal luminal contents. Although particle uptake
1605 across the intestinal epithelium can also be achieved by goblet cells, these cells have been
1606 shown to only take up particles less than 0.02 μm in diameter[429]. Transepithelial dendrites
1607 extended from $CD103^+$ dendritic cells (DC) also take up small particles from the
1608 intestinal lumen[430]; however, there are currently no reports demonstrating that $CD103^+$
1609 DCs take up particles larger than bacteria. Thus, intact probiotic yeast, of average size
1610 between 3-6 μm in diameter, are most likely to be taken up by M cells and transferred to

the Peyer's patches. Described here is a protocol for collection and screening of Peyer's patches for viable recombinant yeast, although this procedure can also be easily adapted for evaluating uptake of probiotic bacteria.

In summary, assessing recombinant probiotic yeast for the delivery of therapeutic proteins to the intestine requires proficiency in laboratory techniques spanning molecular biology to animal handling and immunology. Presented here are protocols for 1) the generation and screening of auxotrophic yeast strains which can be easily negatively selected without antibiotics, 2) alternative protocols to transform yeast and enable expression of heterologous protein, 3) demonstrations of proper animal handling techniques and oral gavage for intragastric delivery of recombinant yeast, and 4) protocols for Peyer's patch dissection and screening for viable recombinant yeast and functional heterologous protein. Combined, these protocols will allow for the generation and testing of a probiotic yeast strain capable of delivering heterologous therapeutic protein to the gastrointestinal tract.

a) UV mutagenesis to generate auxotrophic yeast strains

1.1) Generate survival curves to determine needed doses of UV irradiation

1.1.1) Prepare YPD (yeast extract peptone dextrose) media and other reagents listed in table 1 according to standard procedures[431] and inoculate single colonies into 5-10 mL of YPD media. Incubate cultures on a roller drum at 30 °C overnight to saturation for at least 8 hr.

1632 1.1.2) Determine the cell concentration of overnight cultures using a spectrophotometer
1633 by diluting cells 1:10 in water in a plastic cuvette. Dilute cells to a concentration of 10^7
1634 cells/mL in 20 mL sterile distilled water.
1635 1.1.3) Pour diluted cells into a sterile plastic petri dish and, with the lid removed, place
1636 the plate 14 cm below a UV bulb.
1637 1.1.4) Expose cells to serial 5,000 µJ and 10,000 µJ doses of UV irradiation, extracting
1638 500 µL of cells following each increment such that cells are sampled after exposure to 0
1639 µJ, 5,000 µJ, 10,000 µJ, 15,000 µJ, 20,000 µJ, 25,000 µJ, 30,000 µJ, 40,000 µJ, and
1640 50,000 µJ of UV irradiation. Transfer extracted cell samples to sterile 1.5 mL tubes and
1641 serially dilute at 1:10 increments in sterile water.
1642 1.1.5) Pellet cells in each dilution by centrifugation in a microcentrifuge at 16,000 x g for
1643 1 min. Aspirate supernatant and resuspend in a 100 µl volume of sterile water appropriate
1644 for plating yeast cells. Pipette the full volume of resuspended cells onto plates containing
1645 YPD solid media and use a sterile spreader to evenly distribute cells across each plate.
1646 1.1.6) Wrap plate edges in parafilm to prevent drying of media and cover plates in
1647 aluminum foil to prevent photo-reactivation and repair of UV-induced mutations.
1648 Incubate plates upside down at 30 °C for 2-4 days to allow for growth of viable yeast
1649 colonies (Fig 5.1).
1650 NOTE: Generation of a survival curve following UV irradiation requires plating
1651 of diluted yeast cells such that distinct colony forming units (CFU) are able to form. Each
1652 500 µL sample collected as described above contains approximately 5 x 10^6 cells;
1653 however, greater than 100 colonies per plate are difficult to accurately distinguish.

1654 Plating undiluted sample as well as serial 1:10 dilutions of irradiated cells thus ensures
1655 that CFU can be enumerated at each UV dose, as demonstrated in Fig 5.1.
1656
1657 1.1.7) Count the number of colonies, optionally with the help of a pen to mark off
1658 counted colonies, a hand held electronic counter pen, or a counter stand with
1659 magnification. Plot as a percentage of total plated cells at each μJ dose of UV irradiation
1660 to generate a survival curve for irradiated yeast (Fig 5.2). The CFU count, multiplied by
1661 the dilution factor, is then divided by the total number of original irradiated cells in each
1662 500 μL sample in order to determine percent survival at each dose. Fig 5.2 shows the
1663 calculated percentage of diploid wild type *S. boulardii* cells able to survive 0 μJ, 5,000
1664 μJ, 10,000 μJ, 15,000 μJ, 20,000 μJ, 22,500 μJ, 25,000 μJ, 35,000 μJ, and 50,000 μJ.
1665 These data establish a clear curve that can be used to find the dose corresponding to 50%
1666 survival.
1667 NOTE: Haploid yeast strains can be expected to require lower doses of UV
1668 irradiation relative to diploid strains to reach the same percent survival. A strain of yeast
1669 lacking functional DNA repair enzymes, such as the *rad1 S. cerevisiae* mutant, can be
1670 used as a positive control to indicate the presence of UV irradiation at very low doses.
1671 NOTE: Although such high UV doses increase the risk of mutations in genes for
1672 cellular pathways other than the auxotrophic marker gene of interest, this drawback must
1673 be balanced against the need to induce mutations in both copies of the auxotrophic
1674 marker gene. For haploid strains in which only one gene copy must be mutated, screening
1675 at a higher percent survival, such as at 90%, decreases the risk of additional mutations
1676 and still allows for sufficient generation of auxotrophic mutants.

1677

1678 **b) UV mutagenesis and screening for auxotrophic yeast strains**

1679 1.2.1) Prepare yeast as described in steps 1.1.1-1.1.3.

1680 1.2.2) Expose yeast to the dose of UV irradiation corresponding to 50% survival, as
1681 determined in 1.1.8. For WT *S. boulardii*, this dose was determined to be approximately
1682 18,000 µJ (Fig 5.2).

1683 1.2.3) Collect 1 mL volumes of UV irradiated yeast and pellet by centrifugation in a
1684 microcentrifuge at 16000 x g for 1 min. Aspirate supernatant and resuspend cells in 100
1685 µl sterile water.

1686 1.2.3.1) Selection of mutants

1687 1.2.3.1.1) If using selection such as with *ura3*$^-$ auxotrophic mutants, plate irradiated yeast
1688 onto media containing 5-fluoroorotic acid (5-FOA) to select for cells lacking a functional
1689 Ura3 enzyme.

1690 NOTE: Any yeast containing functional copies of Ura3 will convert 5-FOA to the toxin
1691 5-fluorouracil, leading to cell death and allowing for easy selection of *ura3*$^-$ colonies that
1692 lack a functional Ura3[432]. Analogous selection approaches are possible for the *LYS2* and
1693 *LYS5; TRP1;* and *MET2* and *MET15* markers using media containing α-aminoadipic
1694 acid[433]; 5-fluoroanthranilic acid[434]; and methyl mercury[435,436], respectively.

1695 1.2.3.1.2) Pipette the 100 µl of resuspended cells onto minimal media containing 5-FOA
1696 and use a sterile spreader to evenly coat the plate. Wrap plates in parafilm and incubate
1697 upside down at 30 °C for 2-4 days to allow for growth of viable yeast colonies.

1698 1.2.3.1.3) Confirm the *ura3*$^-$ phenotype of any colony appearing on 5-FOA plates by
1699 restreaking onto YPD, uracil$^-$ and 5-FOA plates (Fig 5.3). Use the tip of a sterile

1700 toothpick to collect part of a single colony and gently drag the cells across fresh YPD,
1701 uracil$^-$, and 5-FOA plates. Again incubate wrapped plates upside down at 30 °C for 2-4
1702 days. Viable colonies will appear as raised, roughly circular growths while non viable
1703 cells will appear only as an opaque smear without any raised growths (Fig 5.3).
1704 NOTE: After selection of UV dose and irradiation of yeast cells, it is critical to
1705 screen mutant colonies to confirm lack of a functional auxotrophic marker gene. Use of a
1706 selection method, as described in 1.2.3.1 and shown in Fig 5.3, significantly increases the
1707 efficiency of phenotype confirmation. Shown is an example of *URA3* selection that takes
1708 advantage of the conversion of 5-FOA to the toxin 5-FU by intact Ura3. Analogous
1709 approaches are available for *LYS2* and *LYS5; TRP1;* and *MET2* and *MET15* and increase
1710 efficiency of selection for these mutations. Care must be taken to select individual
1711 colonies during screening. The consistent growth of mutant colonies on YPD and 5-FOA,
1712 but not uracil$^-$, plates indicates auxotrophic phenotype.
1713 1.2.3.2) Screening of mutants
1714 1.2.3.2.1) If generating an auxotrophic mutant for which selection methods are not
1715 available, plate UV irradiated yeast onto YPD media. Prepare serial 1:10 dilutions of UV
1716 irradiated yeast cells in sterile water and pipette the dilutions onto YPD media, using a
1717 sterile spreader to evenly coat the plate. Wrap plates in parafilm and incubate upside
1718 down at 30 °C for 2-4 days. Determine which dilution allowed for growth of individual
1719 colonies that can easily be distinguished from each other, usually no more than 100
1720 colonies per plate. Repeat UV mutagenesis of yeast samples as described in 1.2.1-1.2.3
1721 and plate cells at this determined dilution. Pipette the diluted yeast onto YPD media, use

1722 a sterile spreader to distribute the cells, and incubate wrapped plates upside down at 30
1723 °C for 2-4 days.
1724 1.2.3.2.2) Screen for auxotrophs by replica plating onto selective media lacking the
1725 metabolite of interest. First, secure a sterile velvet pad onto a plate stand and invert the
1726 plate with UV irradiated colonies onto the velvet, marking the orientation of each plate.
1727 Next, invert a fresh plate lacking the metabolite of interest onto the velvet and lightly
1728 press down to transfer cells from the velvet to the plate. Store the original plate at 4 °C
1729 and incubate the new plate wrapped and upside down at 30 °C for 2-4 days.
1730 1.2.3.2.3) As an alternative to replica plating, screen mutants by restreaking colonies
1731 from YPD onto selective media. Use the tip of a sterile toothpick to collect part of a
1732 single colony and gently drag the cells across fresh YPD plates and plates lacking the
1733 metabolite of interest. Again incubate wrapped plates upside down at 30 °C for 2-4 days.
1734 NOTE: Care must be taken to select single colonies and streak out colonies multiple
1735 times to confirm a homogeneous population of true auxotrophic cells.
1736 1.2.4) Further confirm the phenotype of irradiated cells by inoculating single colonies
1737 into 5-10 mL of both YPD and media lacking the appropriate metabolite (eg. in uracil$^-$
1738 media for a *ura3*$^-$ mutant). Incubate on a roller drum at 30 °C overnight to confirm
1739 growth of cells in YPD media but not in the absence of the metabolite.
1740 NOTE: Although growth patterns on solid media should clearly indicate yeast
1741 auxotrophic status, it is possible for some yeast to tolerate stresses and form small, slow
1742 growing colonies on solid media but yet not tolerate the same conditions in liquid media
1743 (unpublished observations). Inoculation into liquid cultures should thus be performed to
1744 thoroughly confirm growth patterns of UV irradiated mutants.

1.2.5) For long term storage of confirmed auxotrophic mutants, prepare glycerol stocks by inoculating cells into 10 mL YPD and incubating on a roller drum overnight at 30 °C. Pellet cells by centrifugation for 3 min at 2500 x g and aspirate media. Resuspend cells in 50% sterile filtered glycerol, transfer to a cryovial, and store at -80°C.

NOTE: UV mutagenized yeast potentially contain mutations in multiple genes other than in the auxotrophic marker of interest. Before continuing with use of verified auxotrophic mutants, these strains should be further analyzed through gene sequencing and assessment of resistance to pH, bile acid stresses, and other characteristics relevant to probiotic strains, as described elsewhere[408]. Additionally, use of pcr homology or CRISPR/Cas9 targeting to more selectively mutate auxotrophic markers should be considered as an alternative to UV mutagenesis[410–412].

c) Yeast transformation

2.1) LiOAc Transformation of Yeast

2.1.1) Inoculate single yeast colonies into 5-10 mL of YPD media and incubate on a roller drum at 30°C overnight.

2.1.2) To induce log phase growth and increase efficiency of plasmid uptake, determine cell concentration using a spectrophotometer to measure a 1:10 dilution of cells in sterile water in a plastic cuvette. Dilute overnight cultures to an OD_{600} of 0.16-0.2 (approximately 2×10^6 – 2.5×10^6 cells/mL) in 50 mL of fresh warm YPD and incubate cells on an orbital platform shaker set to 200 rpm until the culture reaches approximately 1×10^7 cells/mL, usually around 4 hours.

1767 NOTE: Transformation efficiency can be measured as a function of the number of
1768 successfully transformed yeast colony forming units (CFU) per µg of plasmid DNA.
1769 Increased efficiency results in more transformed colonies per µg of plasmid DNA.
1770 Subculturing yeast cells and collection during log phase growth is one factor that
1771 increases transformation efficiency[417].
1772 2.1.3) Pellet cells by centrifugation at 2500 x g for 3 min.
1773 2.1.4) Aspirate the supernatant and transfer cells to a 1.5 mL microcentrifuge tube by
1774 resuspending the pellet in 1 mL sterile water.
1775 2.1.5) Pellet cells by centrifugation at 16000 x g for 1 min in a microcentrifuge, aspirate
1776 the supernatant and wash cells by resuspension in 1 mL TE/LiOAc (Tris EDTA LiOAc)
1777 buffer.
1778 2.1.6) Repeat centrifugation and resuspend cells in TE/LiOAc buffer to a concentration of
1779 2×10^9 cells/mL.
1780 2.1.7) Prepare transformation mixtures with each of the following: 50 µL of prepared
1781 yeast in TE/LiOAc buffer, 5 µL of carrier DNA (10 µg/µL), and 1 µL of plasmid DNA (1
1782 µg). Micrograms of plasmid DNA may be titrated as increasing amounts of DNA may or
1783 may not lead to increased transformation efficiency[437]
1784 NOTE: For a mutant yeast strain lacking one auxotrophic marker, only one plasmid
1785 encoding the marker can be transformed per sample. Furthermore, use of a plasmid
1786 encoding an easily detectable protein, such as GFP, will allow for efficient determination
1787 of proper folding and expression of heterologous protein by the yeast strain subsequent to
1788 transformation.

1789 2.1.8) To each preparation, add 300 μL of PEG/LiOAc/TE and vortex thoroughly.
1790 Incubation of intact cells with PEG is essential for efficient transformation[418].
1791 2.1.9) Incubate preparations at 30 °C for 30 min with agitation by placing
1792 microcentrifuge tubes in a beaker placed onto an orbital platform shaker at 200 rpm.
1793 2.1.10) Add 35 μL of DMSO to each reaction and heat shock cells for 15 min in a 42 °C
1794 water bath. Although there are conflicting reports of the added benefit of DMSO[438], heat
1795 shock of intact yeast cells has been shown to greatly increase transformation
1796 efficiency[417].
1797 2.1.11) Wash cells by pelleting via centrifugation as in 2.1.5, aspirating or pipetting off
1798 the supernatant, and resuspending in 1 mL sterile water. Gently pipette up and down to
1799 break up the cell pellet.
1800 NOTE: It is critical to thoroughly remove the supernatant because the media used in
1801 generating competent cells can inhibit yeast growth and colony formation.
1802 2.1.12) Repeat cell pelleting as in 2.1.5, aspirate the supernatant, and resuspend the cells
1803 in 100 μl sterile water. Pipette the full volume onto a selective plate and use a sterile
1804 spreader to evenly coat the plate with transformed yeast.
1805 2.1.13) Wrap edges of coated plates in parafilm to prevent drying of media and incubate
1806 upside down at 30 °C for 2 days to allow for growth of transformed yeast cells.
1807 Successful, efficient transformation and auxotrophic selection of *Saccharomyces*
1808 *cerevisiae* yields a high number of colonies per transformation preparation, although
1809 yield can be much lower for other strains (Fig 5.4).
1810 **NOTE:** Fig 5.4 shows transformation efficiency for wild type *S. boulardii* (*S.b.*)
1811 relative to a commonly used laboratory *S. cerevisiae* strain (*S.c.*) using both the LiOAc

1812 (LiOAc) and electroporation (Electro) techniques. Although LiOAc transformation is
1813 very efficient for *S. cerevisiae*, transformation efficiency for *S. boulardii* is greatly
1814 improved using electroporation.
1815 2.1.14) Store yeast plates for the short term (generally 1-3 weeks) upside down and
1816 covered at 4 °C. Prepare glycerol stocks of transformed yeast as described in 1.2.5 for
1817 long term storage.
1818 NOTE: Further studies testing transformed CFU are necessary to determine
1819 plasmid stability and evaluate proper expression of heterologous protein. Thorough
1820 descriptions of plasmid stability[439], use of immunoblotting to detect denatured proteins
1821 recovered from cell samples[422], enzyme linked immunosorbant assay (ELISA) to detect
1822 properly folded three dimensional proteins [421], and the use of GFP in yeast studies[440] are
1823 available elsewhere. Fig 5.5 shows a representative image of successful GFP expression
1824 in transformed *S. cerevisiae* relative to untransformed cells. Use of such a fluorescent
1825 protein is one means of efficiently determining successful heterologous protein
1826 production.
1827 **2.2 Electroporation of Yeast**
1828 2.2.1) Inoculate single yeast colonies into 5-10 mL of YPD media and incubate on a
1829 roller drum at 30 °C overnight.
1830 2.2.2) Determine cell concentration using a spectrophotometer to measure a 1:10 dilution
1831 of cells in sterile water. Dilute overnight cultures in 100 mL fresh warm YPD media to an
1832 OD_{600} equivalent of approximately 0.3. Incubate subcultures at 30 °C on an orbital
1833 platform shaker set to 200 rpm until reaching an OD_{600} of approximately 1.6, usually 4-5

1834 hours. Each 100 mL subculture will generate enough conditioned cells for two
1835 transformation reactions.
1836 2.2.3) Pellet cells by centrifugation at 2500 x g for 3 min. Aspirate the supernatant and
1837 wash cells by resuspending in 50 mL ice cold sterile water. Repeat the wash by pelleting
1838 cells, aspirating supernatant, and resuspending in fresh 50 mL ice cold sterile water.
1839 2.2.4) Pellet the cells again and resuspend in 50 mL ice cold electroporation buffer (1 M
1840 Sorbitol, 1 mM $CaCl_2$).
1841 2.2.5) Repeat spin as in 2.2.3, aspirate supernatant, and resuspend cells in 20 mL 0.1 M
1842 LiOAc/10 mM DTT. Incubate cell suspension on a roller drum at 30 °C for 30 min.
1843 Preincubation of cells in LiOAc and DTT synergistically increases the efficiency of
1844 electroporation[441].
1845 2.2.6) Pellet the cells as in 2.2.3, remove supernatant, and wash by resuspending in 50
1846 mL ice cold electroporation buffer. Repeat centrifugation and resuspend cells in ice cold
1847 electroporation buffer to a final volume of 1 mL.
1848 2.2.7) Prepare on ice: conditioned yeast cells, sterile electroporation cuvettes, and
1849 plasmid DNA. Immediately after final resuspension of conditioned cells in 1 mL
1850 electroporation buffer, combine 400 µl conditioned yeast cells with approximately 1 µg
1851 of plasmid DNA and add to an ice cold 0.2 µm electroporation cuvette. Use of increased
1852 amounts of DNA may slightly increase transformation efficiency[416]. Incubate reaction on
1853 ice for 5 min, then electroporate with electroporator set to 2.5 kV and 25 µF.
1854 NOTE: As described in 2.1.7, a mutant yeast strain lacking one auxotrophic
1855 marker can be transformed with only one plasmid encoding the mutated marker per
1856 sample. Also, using a plasmid that encodes an easily detectable protein such as GFP

1857 allows for efficient determination of proper folding and expression of heterologous
1858 protein subsequent to transformation.
1859 2.2.8) Transfer electroporated cells into 8 mL of a 1:1 mixture of YPD:1 M sorbitol and
1860 allow cells to incubate on a roller drum at 30 °C for 60 min.
1861 2.2.9) Pellet cells as in 2.2.3 and resuspend in 100 µl 1:1 YPD:1 M sorbitol. Plate the full
1862 volume onto selective media containing 1 M sorbitol, wrap edges of plates in parafilm,
1863 and incubate plates upside down at 30 °C for 2-5 days to allow for growth of transformed
1864 yeast cells.
1865 NOTE: It is critical to test transformed yeast to evaluate proper expression of
1866 heterologous protein, as described in 2.1.14 and 2.2.7.
1867

1868 **d) Oral gavage of mice with transformed yeast**

1869 3.1) Perform all animal care and handling procedures according to the Guide for the Care
1870 and Use of Laboratory Animals and Institutional Animal Care and Use Committee
1871 approval.
1872 3.2) Prepare overnight yeast cultures by inoculating single colonies of transformed
1873 auxotrophic yeast into 5-10 mL of selective media. Incubate cultures overnight for at
1874 least 8 hr on a roller drum at 30 °C until saturated.
1875 NOTE: Use of plasmid encoding test proteins such as GFP will allow for ease of
1876 protein expression testing in gavaged yeast, as described in 4.7. Fig 5.5 shows use of
1877 fluorescence microscopy as an example method of analyzing proper protein expression
1878 from transformed yeast. Brightfield (A) and fluorescence (B) images are shown for *S.*
1879 *cerevisiae* transformed with a URA3 plasmid encoding GFP, demonstrating functional

1880 expression of heterologous protein from the transformed yeast. Cells can be immobilized
1881 for better visualization using coverslips coated in concanavalin A (coat 5 µL of a 2 mg/ml
1882 stock solution in water onto each 22 x 22 µm coverslip and air dry).
1883 3.3) For maximal induction of protein expression and to induce log phase growth, prepare
1884 subcultures from the overnight cultures by diluting to an OD_{600} equivalent of
1885 approximately 0.16-0.2 in 50 mL of appropriate media as described in 2.1.2.
1886 3.4) Determine the concentration of subcultured cells as in 1.1.2 and adjust to 10^9
1887 cells/mL. Prepare a 100 µl dose for each mouse, with a minimum 500 µl volume per
1888 group to improve accuracy and ease of sample loading.
1889 3.5) Pellet cells by centrifugation at 2500 x g for 3 min or in a microcentrifuge at 16000 x
1890 g for 1 min. Aspirate supernatant and resuspend cells by adding an equal volume of
1891 sterile water and gently pipetting up and down.
1892 3.6) Fix an appropriate gauge gavage needle (22 gauge for 15-20 g mice) onto a 1 mL
1893 sterile syringe and load yeast sample, being sure to eliminate any bubbles and set plunger
1894 to a 100 µL increment. Load an additional syringe with sterile water to gavage control
1895 mice and check for presence of any contaminating yeast.
1896 3.7) Pick up the mouse to be gavaged using the non dominant hand, with index finger and
1897 thumb tightly grasping the skin around the neck (Fig 5.6A). Tuck the tail under the small
1898 finger to prevent movement of the lower body. Be sure that the grip is secure and
1899 prohibits the mouse from moving its head in order to prevent damage to internal tissues
1900 during gavage. Estimate how far the gavage needle should be inserted by holding the
1901 needle against the mouse such that the bulb is even with the xiphoid process of the

1902 sternum. Inserting this length of the needle will allow the gavage needle bulb to enter the
1903 stomach.
1904 3.8) Using the dominant hand, gently insert the gavage needle into the mouse esophagus
1905 by angling the needle along the roof of the mouth and back of the throat, keeping slightly
1906 to the left of center. Wait for the mouse to swallow the bulb of the needle and allow
1907 needle to descend slightly further to the point estimated in 3.7 (Fig 5.6B). If any
1908 resistance is felt during insertion of the gavage needle or if the mouse at any time begins
1909 to gasp, gently remove the needle and again try to find the esophagus.
1910 3.9) After the mouse has swallowed the bulb of the gavage needle, gently depress the
1911 syringe plunger to administer 100 µl (10^8 CFU) of yeast directly into the mouse stomach.
1912 NOTE: Although mice are unable to vomit any of the gavaged solution after
1913 administration[423,424], it is possible for reflux to occur during gavage[426,442]. Proper
1914 insertion of the gavage needle, as well as adjusting the volume and viscosity of the
1915 solution, can help to limit reflux and ensure accurate dosing.
1916 3.10) Carefully remove the gavage needle from the mouse stomach and esophagus and
1917 return the mouse to the cage. Check that the mouse is breathing and moving normally
1918 after gavage to ensure that the gavage needle was properly inserted throughout the
1919 procedure and that no solution was aspirated.
1920

1921 ### e) Harvest of murine Peyer's patches and isolation of viable yeast
1922 ### colonies

1923 4.1) At the appropriate time point post gavage, typically four hours, sacrifice mice using
1924 IACUC approved methods such as by isoflurane euthanasia. Add isoflurane to absorbent

1925 gauze at the bottom of a container with an airtight lid and cover the gauze with paper
1926 towels such that the mouse will not directly contact the anesthetic. Put the mouse in the
1927 container, seal the lid, and wait until the mouse is no longer responsive. Check for lack of
1928 responsiveness following a toe pinch, and use a secondary measure such as cervical
1929 dislocation to sacrifice the mouse. Additional time points may also be tested, as
1930 numerous studies have shown that efficiency and timing of uptake across the epithelium
1931 is particle dependent[443,444].
1932 4.2) Lay the mouse with the abdomen fully exposed and sterilize the abdominal area by
1933 spraying with 70% EtOH. Make a longitudinal incision through the fur and skin with
1934 scissors, being careful not to damage any internal tissues. Manually pry the incision open
1935 further to expose the peritoneum, the thin serosal lining covering the abdominal organs.
1936 Gently lift the peritoneum and make a longitudinal incision to expose the intestines.
1937 4.3) Carefully use blunt forceps to tease the small intestine away from the mesenteric
1938 arteries, fat, and other tissues. Expose the small intestine from the stomach, in the upper
1939 left quadrant of the mouse abdomen, to the cecum, the large pocket of intestinal tissue at
1940 the start of the large intestine.
1941 4.4) Isolate the Peyer's patches by looking for 1-3 mm roughly circular patches of opaque
1942 tissue along the small intestine (Fig 5.7). Most mice have between 4-8 easily visible
1943 Peyer's patches. Performing the procedure in an area with direct overhead lighting will
1944 increase the ease with which Peyer's patches can be visualized. Using curved dissection
1945 scissors, cut away the dome of the Peyer's patch, leaving margins to ensure that none of
1946 the surrounding lamina propria is collected.

1947 4.5) Collect dissected Peyer's patches in complete Iscove's modified dulbecco's media
1948 (IMDM). It is critical to use sterile technique and include antibiotics in the collection
1949 media in order to prevent gastrointestinal bacterial contamination of yeast plates.
1950 4.6) Strain Peyer's patches through a 40 μm cell strainer to eliminate collection media.
1951 Wash Peyer's patches with fresh complete IMDM over a 50 mL tube and use a plunger
1952 from a 1 mL syringe to gently break up the Peyer's patches. Pellet strained cells by
1953 centrifugation at 1800 rpm for 7 min. Aspirate supernatant and resuspend cells in a final
1954 volume of approximately 100 μL.
1955 4.7) Apply strained cells onto selective yeast media and use a plate spreader to evenly
1956 distribute cells. Wrap plate edges in parafilm and incubate plates upside down at 30 °C
1957 for 2 days to allow for growth of any viable yeast recovered from the murine Peyer's
1958 patches, as shown in Fig 5.8.
1959 NOTE: Further studies after recovery of yeast from Peyer's patches are necessary
1960 to confirm that the strains are able to deliver properly folded heterologous protein to these
1961 immune tissues. As described in 2.1.14, such methods may include immunoblotting,
1962 ELISA, or fluorescence microscopy to detect fluorescent proteins such as GFP[408,445].
1963

1964 **f) Discussion**

1965 Together, the protocols herein describe the essential steps necessary for the
1966 development and testing of auxotrophic probiotic yeast strains for delivery of
1967 heterologous therapeutic protein to the intestine. This manipulation and testing of
1968 recombinant probiotic yeast requires techniques and resources with which any individual
1969 laboratory may not currently be familiar. Thus, although numerous previous studies have

1970 described the above protocols for multiple yeast and mouse strains, these methods have
1971 not to the authors' knowledge been presented in a detailed, unified form. Furthermore,
1972 the present manuscript places particular emphasis on adapting current standardized
1973 protocols for the genetic manipulation of probiotic yeast, which are less well
1974 characterized than commonly used laboratory yeast strains. Many steps for both
1975 mutagenesis (discussed in part 1) and transformation (part 2) must be optimized for the
1976 manipulation of such diploid, probiotic yeast isolates. This section also discusses
1977 potential pitfalls associated with animal handling (part 3) and dissection of the Peyer's
1978 patch immune tissues of the small intestine (part 4).

1979 As many industrial and clinically relevant yeast strains are not immediately
1980 adaptable to large scale genetic manipulation, it is first necessary to generate strains such
1981 as auxotrophic mutants that can be grown and selected without expensive antibiotics. UV
1982 mutagenesis is one such approach that allows for quick nonspecific mutation of
1983 auxotrophic genes[332,446]. Survival curves can easily be generated (Figs 5.1 and 5.2) to
1984 determine the appropriate dose for screening mutants. However, this approach carries the
1985 risk of inducing off target mutations that may affect growth rate or other properties of the
1986 yeast strain. Targeted knockouts can instead be generated using PCR constructs or the
1987 CRISPR/Cas9 system. Subsequent screening or selection (Fig 5.3) of mutants allows for
1988 identification of auxotrophic yeast. Use of selection by plating onto 5-FOA media, for
1989 example, allows for rapid elimination of any yeast still containing a functional *URA3*
1990 auxotrophic gene. When possible, this selection approach may be preferable to a screen,
1991 which requires analysis of all colonies generated. With either selection or screening,

1992 however, it is critical to perform repeated streaking of individual yeast colonies onto
1993 selective media to confirm auxotrophic status.

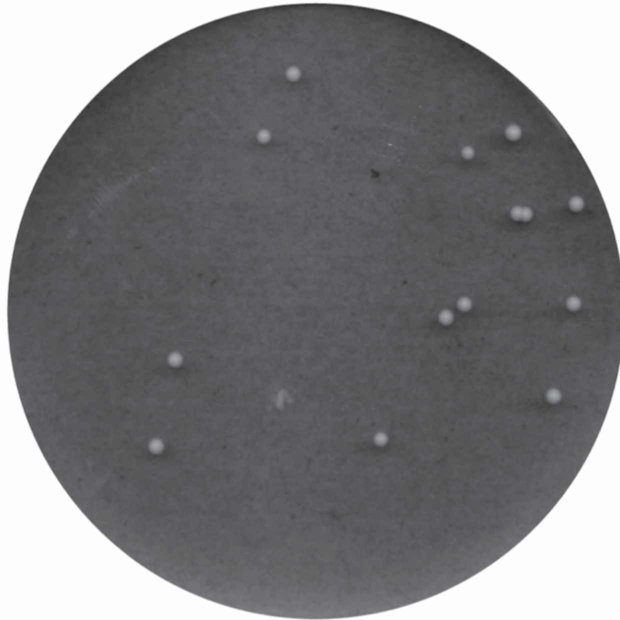
1994 Transformation of the generated mutants can be accomplished through different
1995 protocols. Although LiOAc transformation is effective in the transformation of many
1996 yeast strains, particularly for the most commonly used laboratory *S. cerevisiae* strains,
1997 alternative protocols such as electroporation may transform other yeast isolates with
1998 greater efficiency (Fig 5.4). Each new strain should be tested using multiple protocols to
1999 determine the optimal conditions for transformation. Varying incubation times and
2000 concentration of DNA, for example, can influence overall transformation efficiency and
2001 should be tested and optimized for each strain[438].

2002 Oral gavage allows for the delivery of controlled doses of these recombinant yeast
2003 directly to the murine gastrointestinal tract, whose immune tissues can then be assayed
2004 for yeast and heterologous protein. Proper oral gavage technique (Fig 5.6) is critical to
2005 minimize animal discomfort and increase experimental precision. Furthermore, the
2006 Peyer's patches are key sites to assess uptake of recombinant yeast from the intestine.
2007 These clusters of immune tissue are important sites of antigen sampling and induction of
2008 mucosal immune responses. Large antigens, including yeast 3-6 μm in diameter, are most
2009 likely to be taken up by the M cells of Peyer's patches in order to cross the
2010 gastrointestinal epithelium and interact with immune cells. Care must be taken when
2011 dissecting the Peyer's patches to ensure that only cells from within the patch rather than
2012 the intestinal lumen or lamina propria are collected (Fig 5.7). Further steps must also be
2013 taken following dissection to assess proper expression and function of heterologous
2014 protein in the recovered yeast (Fig 5.8). Preparation of total protein from yeast lysates

2015 and immunoblotting is one standard method to assess protein expression; however, this
2016 approach does not provide information regarding protein folding and function. To assess
2017 protein function, yeast can be transformed with a plasmid encoding GFP and analyzed
2018 under a fluorescent microscope after recovery from Peyer's patches to assess functional
2019 GFP expression (Fig 5.5).

2020 In sum, this manuscript presents a unified set of detailed experimental protocols
2021 spanning steps from the generation of auxotrophic mutants to the recovery of probiotic
2022 yeast from the murine intestine. By compiling protocols that do not traditionally fall
2023 within a single area of expertise, these descriptions will facilitate further studies testing
2024 immunological responses to probiotic yeast designed as oral drug delivery vectors. The
2025 authors hope this study will encourage discussion and promote optimization of
2026 experimental methods for each yeast strain tested, paving the way for the most efficient
2027 approaches to the development of novel, probiotic-based recombinant therapies.
2028

2029 **g) Figures and Tables**



2030

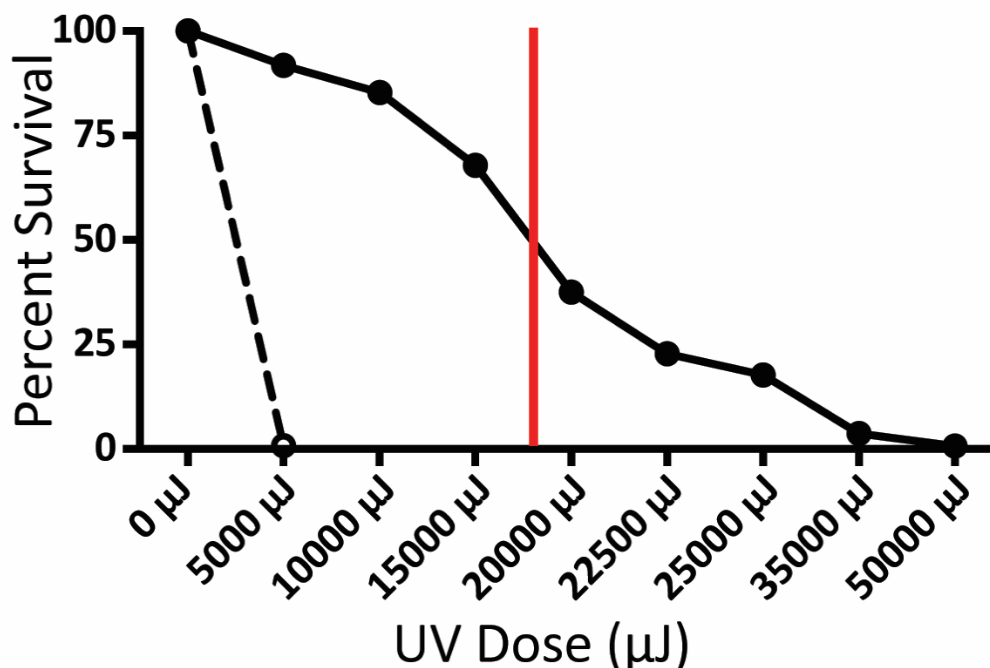
2031 **Fig 5.1 Yeast colonies grown on YPD media**

2032 Example YPD plate showing viable colony forming units (CFU) of probiotic yeast after

2033 UV irradiation. Cells were serially diluted such that individual CFU can be distinguished

2034 and counted.

2035



2036

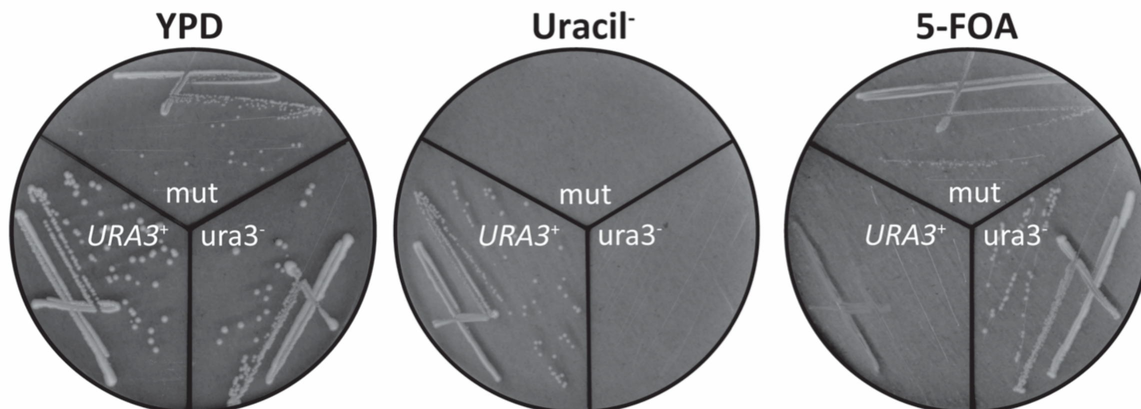
2037

2038 **Fig 5.2 Survival curve for diploid probiotic yeast**

2039 Number of viable *S. boulardii* CFU as a percent of total plated cells was plotted for each
2040 µJ dose of UV irradiation (solid line). The vertical red line indicates the µJ UV dose
2041 corresponding to 50% survival of this yeast strain. A *rad1 S. cerevisiae* mutant, which
2042 cannot repair damage from UV mutagenesis, is shown as a control (dashed line).

2043 1.1.8) Determine the dose of UV mutagenesis to be used for screening by referring to the
2044 survival curve established in 1.1.7. The x value of the point along the survival curve
2045 where y equals 50 is the UV irradiation dose at which 50% of yeast survive. Screening
2046 mutants at this low percent survival may result in a higher yield of successfully mutated
2047 strains, particularly for diploid yeast. The 50% survival dose for WT *S. boulardii*, as
2048 shown in Fig 5.2, is approximately 18,000 µJ.

2049



2050

2051 **Fig 5.3 Confirmation of *ura3*⁻ phenotype of UV irradiated cells on YPD, uracil⁻, and**

2052 **5-FOA plates**

2053 Cells from individual UV mutant colonies were collected using the tip of a sterile

2054 toothpick and gently streaked across YPD, uracil$^-$, and 5-FOA plates. Cells were first

2055 streaked in two perpendicular crossing lines, then a new toothpick was used to pass

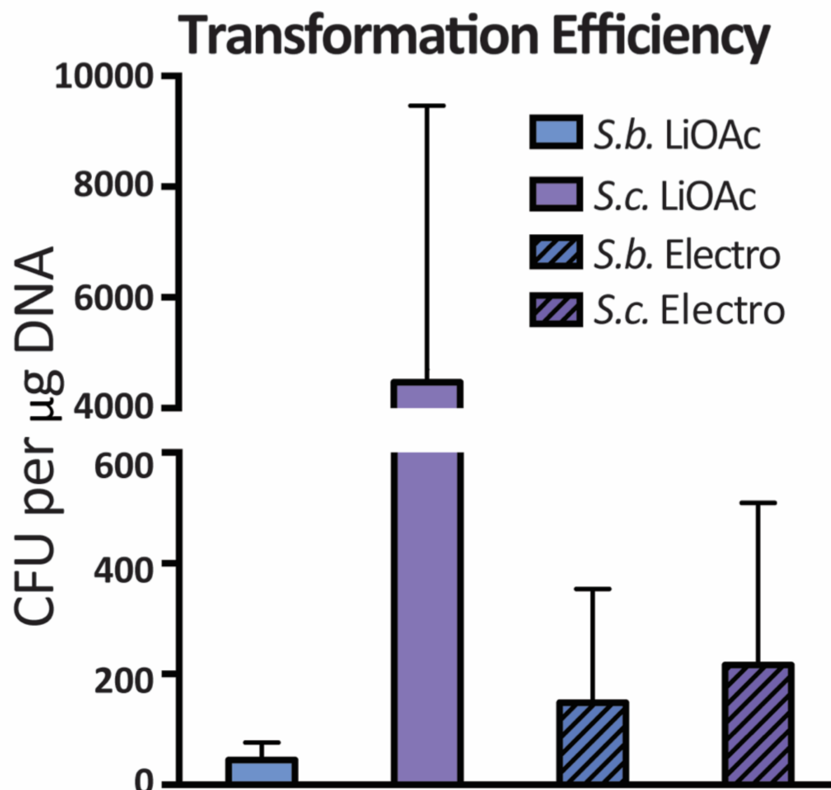
2056 through the second line and continue spreading cells until individual cells separate. A true

2057 *ura3*$^-$ mutant (mut) grows on YPD media and in the presence of 5-FOA, but not in the

2058 absence of uracil. Control *ura3*$^-$ *S. cerevisiae* (*ura3*$^-$) and *URA3*$^+$ *S. boulardii* (*URA3*$^+$)

2059 are shown for comparison and to confirm proper preparation of yeast media.

2060



2061

2062 **Fig 5.4 Transformation Efficiency of *Saccharomyces* strains**

2063 Wild type *S. boulardii* (*S.b.*) and a laboratory *S. cerevisiae* strain (*S.c.*) were transformed

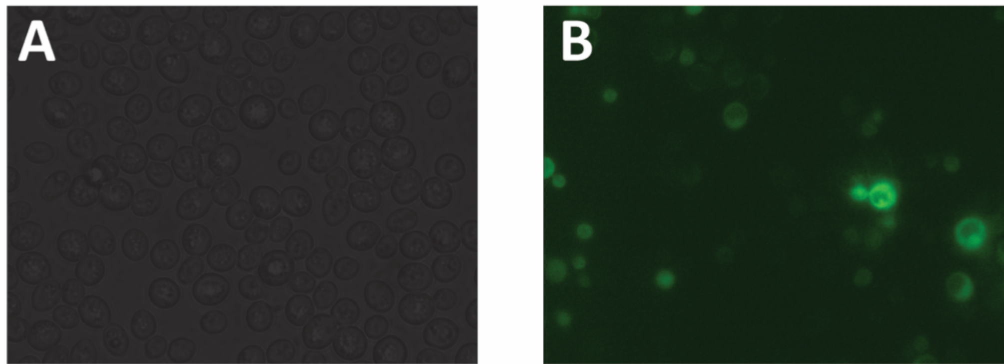
2064 using the described LiOAc (LiOAc) and electroporation (Electro) protocols. Results are

2065 plotted as mean CFU obtained per µg of plasmid encoding a kanamycin resistance

2066 marker. Bars show the mean of duplicate experiments with error bars depicting the

2067 standard error of the mean.

2068



2069

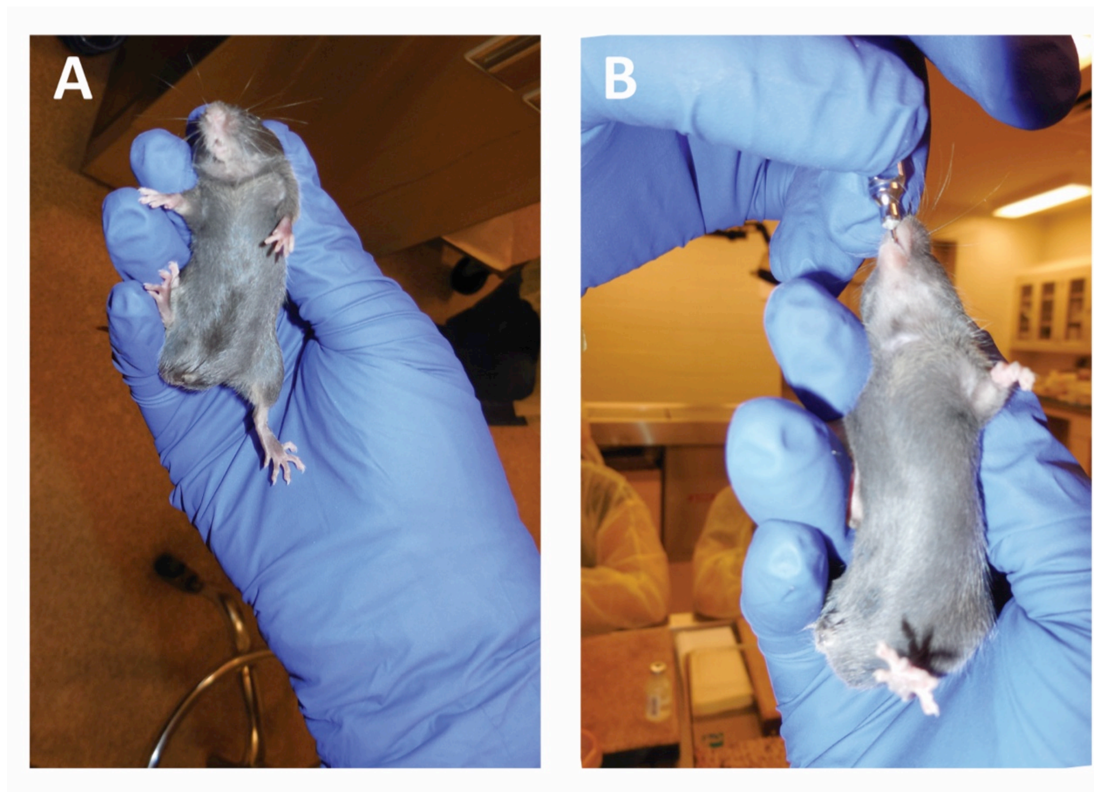
2070 **Fig 5.5 Functional Protein Expression by Transformed Yeast**

2071 *S. cerevisiae* transformed with empty plasmid (5A) and plasmid encoding GFP (5B) were

2072 analyzed using a fluorescent microscope. Fluorescence in the yeast cells transformed with

2073 GFP plasmid indicates successful production of functional GFP.

2074



2075

2076 **Fig 5.6 Proper handling of a C57BL/6 mouse for oral gavage**

2077 A C57BL/6 mouse held just prior to oral gavage (A). The mouse is held tightly in the non
2078 dominant hand grasping the back and neck of the mouse firmly with the tail tucked under
2079 the small finger so that the mouse is not able to move the head in any direction. This hold
2080 allows the gavage needle to be placed accurately and with decreased risk of tissue
2081 damage. The gavage needle is inserted into the pharynx along the roof of the mouth (B).
2082 The mouse is allowed to swallow the bulb of the gavage needle, allowing the solution to
2083 then enter the stomach as the plunger is depressed.

2084

2085

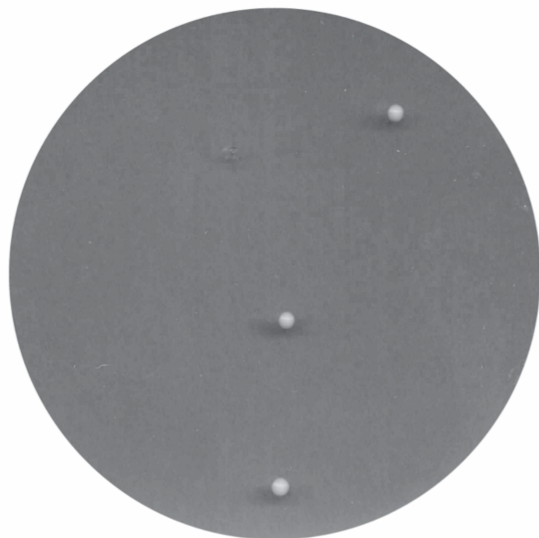
2086 **Fig 5.7 Preparation and dissection of Peyer's patches**

2087 The small intestine is shown dissected away from the other internal organs and tissue,

2088 with arrows pointing to a few of the Peyer's patches.

2089

2090



2091

2092 **Fig 5.8 Yeast Recovery from Peyer's Patches**

2093 An example of viable CFU detected after dissection, homogenization, and plating of total
2094 Peyer's patch cells from a mouse gavaged with *S. boulardii*. Cells were plated onto YPD
2095 yeast media and incubated at 30 °C for 2 days. Typical yield of CFU recovered per
2096 mouse is less than 10.

2097

2098 **Table 5.1 Reagents List**

Solutions	**Yeast Media and Plates**	**Transformation Reagents**
Polyethylene glycol (PEG) 50%: 250 g PEG 3350 500ml sterile water Filter sterilize	YPD: 20 g peptone 20 g dextrose 10 g yeast extract 1 L water Autoclave	TE/LiOAc: 50 ml 10x TE 50 ml 10x (1M) LiOAc 400 mL sterile water Filter sterilize
TE 10X: 100 mM Tris 10 mM EDTA pH to 7.5 and filter sterilize	YPD plates: 20 g peptone 20 g dextrose 20 g bacteria agar 10 g yeast extract 1 L water Autoclave	PEG/TE/LiOAc: 400 ml 50% PEG 50 ml 10x TE 50 ml 10x (1M) LiOAc
20% glucose: 200g dextrose 1 L water Filter sterilize	Uracil^{-} selective media 2 g amino acid mix lacking uracil 6.7 g yeast nitrogen base without amino acids 1 L water Sterilize by autoclaving or sterile filtering Add 20% glucose 1:10 before use	Carrier DNA (SS DNA): Store at -20 and prior to use heat for 1-2min at 100 degree hot plate to melt strands and store on ice

<table>
<tr><td>50% glycerol:
500 mL glycerol
500 mL water
Autoclave</td><td>Uracil⁻ plates:
In a 250 mL flask:
2 g amino acid mix lacking uracil
6.7 g yeast nitrogen base without amino acids
150 mL water
In a 2 L flask:
20 g bacto agar
750 mL water
Autoclave flasks separately, then mix together with 100 mL 20% glucose</td><td>Electroporation buffer:
1 M Sorbitol
1 mM $CaCl_2$
Fill with distilled water
Autoclave and store at 4°C</td></tr>
<tr><td>Complete IMDM
500 mL Iscove's Modified Dulbecco's Media
2.5 mL
5 mL penicillin streptomycin
L-glutamin
HEPES
500 µL mercapto-ethanol</td><td>5-FOA⁺ plates:
Autoclave in a 2 L flask:
20 g bacteria agar
750 mL water
Mix:
6.7 g yeast nitrogen base without amino acids
2 g amino acid mix without uracil
150 mL warm water
When cool, add:
0.05 g uracil powder
1 g 5-FOA</td><td>LiOAc/DTT
0.1 M LiOAc
10 mM DTT</td></tr>
</table>

	Stir and filter sterilize Add to autoclaved agar solution Mix with 100 mL 20% glucose	

2099

2100 Table 5.1 Described are the reagents needed for making each of the solutions, yeast

2101 media and plates, and transformation buffers used for the protocols in this manuscript.

2102

6) Characterization of the Probiotic Yeast *Saccharomyces boulardii* in the Healthy Mucosal Immune System

The following is adapted from the manuscript by Lauren E. Hudson, Courtney D. McDermott, Taryn P. Stewart, William H. Hudson, Daniel Rios, Milo B. Fasken, Anita H. Corbett, and Tracey J. Lamb published in 2016 in *PLOS ONE* (doi:10.1371/journal.pone.0153351).

Figures and data analysis of genomic sequencing and RNA-seq were performed by Will Hudson. Daniel Rios prepared and imaged the Peyer's patch histology.

a) Introduction

Use of viable microorganisms to synthesize and deliver therapeutics directly to the mucosa is an intriguing potential means of treating and preventing gastrointestinal disorders. Numerous studies have investigated the use of probiotic bacteria for the delivery of gastrointestinal therapeutics; however, eukaryotic probiotics have been less well studied. A major advantage of using probiotic yeast for this application is their ability as eukaryotes to create post-translational modifications that might enable expression of a wide variety of therapeutic proteins in their proper conformation. A limited number of *Saccharomyces cerevisiae* strains, particularly *S. cerevisiae* subspecies *boulardii* isolates, have been identified as candidates for this novel therapeutic approach due to their ability to easily express heterologous antigen as well as their current use in treatment of gastrointestinal disorders [332,408].

2125 *S. boulardii* probiotic yeast isolates have already been extensively studied in
2126 terms of their ability to limit inflammation and infection in the gastrointestinal tract [447].
2127 However, there is currently a paucity of information regarding the effects of *S. boulardii*
2128 in the healthy, uninflamed intestine. Effects of probiotics observed in the context of
2129 inflammation or dysbiosis are likely to be heavily influenced by intestinal barrier
2130 breakdown and increased exposure of probiotics to host cells, increased recruitment of
2131 inflammatory immune cells to the intestine, or interactions of probiotics with an altered
2132 microbiota composition [448]. Use of *S. boulardii* in oral vaccine delivery or prophylaxis
2133 entails administration to the healthy host mucosa. The tolerogenic nature of the healthy
2134 intestine may affect not only the level but also the nature of the interactions between
2135 probiotics and the host. The extent of these interactions will have significant implications
2136 for the design and dosing of engineered probiotic yeast for use in disease prevention,
2137 making it crucial to understand the interactions of *S. boulardii* with the healthy host
2138 mucosa in the absence of infection or inflammation.

2139 In the healthy intestine, microorganisms and antigens are largely sequestered
2140 within the center of the lumen, separated from the intestinal epithelium by thick layers of
2141 mucus, antimicrobials, and antibodies [449,450]. In order for *S. boulardii* to successfully
2142 deliver therapeutic proteins to the mucosal immune system, it must overcome these
2143 barriers and reach antigen-sampling cells along the epithelial layer. Goblet cells and
2144 dendritic cells (DCs) take up small particles from the intestinal lumen [430,451]; however, the
2145 host cells most likely to take up large particles such as intact yeast are the microfold (M)
2146 cells of the small intestinal Peyer's patches (PP). These cells transcellularly transfer
2147 antigen from the intestinal lumen to the PP dome, where numerous antigen presenting

2148 cells can take up antigen and induce local immune responses as well as traffic to the
2149 draining mesenteric lymph nodes (MLN) to stimulate further responses [428]. However,
2150 contact with these antigen sampling sites may risk the induction of immune responses
2151 against *S. boulardii* itself. Such immune responses could sequester and clear subsequent
2152 incoming yeast or risk induction of gastrointestinal inflammation upon repeated
2153 administration.

2154 Immune recognition of *S. boulardii* is most likely mediated by the cell wall, a
2155 highly complex structure that mediates responses to external stresses including anaerobic
2156 conditions as well as pH and osmotic changes [452–454]. The cell wall contains many
2157 immunomodulatory components. Mannoproteins, for example, compose the outer layer
2158 of the yeast cell wall and bind galectin 3, DC-SIGN, TLR4, and others [455]. β-glucans,
2159 which constitute the middle layer, ligate Dectin-1 and TLRs 2 and 6 and can stimulate
2160 Langerin positive DCs in small intestinal Peyer's patches [455]. Chitin, a minor component
2161 of the innermost cell wall layer, binds the mannose receptor [456–458]. Indeed,
2162 administration of yeast cell wall fragments such as β-glucans has been found to stimulate
2163 mucosal immune responses and recapitulate some effects of whole probiotics [459–461].

2164 Previous reports of secretory IgA induction after *S. boulardii* administration
2165 [132,135,137] suggest that *S. boulardii* might induce adaptive immune responses. However,
2166 there have been no reports measuring *S. boulardii*-induced changes in healthy systemic
2167 antibody levels or anti-*S. boulardii* antibodies in specific-pathogen-free (SPF) mice.
2168 Furthermore, few studies have examined cell signaling pathways and cytokines induced
2169 by *S. boulardii* in the healthy intestine. The goal of the present study is thus to elucidate
2170 intrinsic and immunomodulatory properties of the probiotic yeast *S. boulardii* in the

2171 healthy intestine. A thorough understanding of these interactions is crucial as they may
2172 affect functions of *S. boulardii* in prophylaxis and as a delivery vector for therapeutics to
2173 the healthy gastrointestinal tract. Our results indicate that *S. boulardii* has a limited
2174 ability to induce immune responses in the healthy mucosa. This suggests that observed
2175 prophylactic effects of administration of this probiotic yeast are not mediated via effects
2176 on the mucosal immune system.
2177

2178
b) Materials and Methods

2179
1) Yeast Strains

2180 *S. boulardii* (Ultra Levure®, American Type Culture Collection® Number:
2181 MYA-797™) was used in all imaging, *in vitro*, and *in vivo* studies. *S. cerevisiae* W303
2182 and BY4741 are well characterized laboratory haploid strains (http://yeastgenome.org/)
2183 used in EM imaging.

2184
2) Yeast Genomic Sequencing and Analysis

2185 Yeast genomic DNA was prepared using the ZR Fungal/Bacterial DNA MiniPrep
2186 kit (Zymo Research). Sequencing was performed by the Emory University Genomics
2187 Core on an Illumina HiSeq 2000 with 100 bp paired end reads. Velvet (version 1.2.10)
2188 was used for *de novo* assembly of contigs. The *S. boulardii* ATCC MYA-797 draft
2189 genome has been submitted as an NCBI Whole Genome Shotgun (WGS) project under
2190 accession number LRVB00000000. SyMap was used to detect synteny between the
2191 sequenced *S.* b*oulardii* draft genome and the *S. cerevisiae* reference genome (R-64-1-1,
2192 accessed via *Ensembl* [462]). SyMap and MUSCLE were used to generate the three-way
2193 alignments between the contigs reported here, the *S. cerevisiae* reference genome, and the

2194 previously reported *S. boulardii* EDRL genome [463,464]. Gene ontology enrichment was

2195 performed at the *Saccharomyces* genome database (http://www.yeastgenome.org/) [465].

2196 **3) Yeast Cell Wall Analyses**

2197 Yeast were grown to saturation in normal YPD media (1% yeast extract, 2%

2198 peptone, 2% glucose/dextrose in distilled water), cryopreserved according to standard

2199 protocols and imaged using a Hitachi H7500 TEM by the Emory Robert P. Apkarian

2200 Integrated Electron Microscopy Core. Cell wall layers were measured using Image J

2201 software, taking the average measurements of 23 cells per strain. Statistics were

2202 calculated using GraphPad Prism 6 software and the Kruskal-Wallis and Dunn's multiple

2203 comparisons tests. For caspofungin assays, yeast grown overnight in normal YPD media

2204 were diluted to 10^7 cells per 200 µL in YPD media adjusted to acidic (pH 4) or basic (pH

2205 8) conditions and containing a 0, 2, 4, or 6 nM concentration of caspofungin diacetate

2206 (Sigma). Control yeasts were also grown in untreated media at approximately pH 6, and

2207 assays were performed in triplicate. OD_{600} readings were taken over 24 hours incubation

2208 at 37°C. The phenol sulfuric acid assay was used to determine relative concentration of

2209 total cell wall monosaccharide content of 10^9 yeast grown to saturation in either normal

2210 YPD media or media containing 6 nM caspofungin as previously described [466,467].

2211 **4) Animal studies**

2212 Female C57BL/6 mice aged 4-6 weeks were obtained from Jackson Laboratories

2213 and maintained in sterile housing conditions. Studies were conducted according to the

2214 Guide for the Care and Use of Laboratory Animals of the National Institutes of Health

2215 and with the approval of the Emory University Institutional Animal Care and Use

2216 Committee (protocol number 2002655). For experiments with fluorescently-labeled *S.*

2217 *boulardii*, mice were gavaged as described [468] with 10^8 CFU of carboxyfluorescein
2218 succinimidyl ester (CFSE) surface-labeled *S. boulardii,* and PP were harvested 0, 0.5, 1,
2219 or 2 hours later. Treatment groups in subsequent experiments were gavaged daily with
2220 10^8 CFU of *S. boulardii* resuspended in 100 µL sterile 1X PBS (Life Technologies),
2221 while naïve controls were gavaged with an equal volume of sterile PBS. Blood samples
2222 were collected by cheek bleeds into heparinized tubes and spun at 17,000 x g in a
2223 microcentrifuge for 5 min at 4°C to collect serum. Fresh fecal pellets were collected,
2224 weighed, and resuspended in 10 fold w/v PBS 2 mM EDTA containing a 1:100 dilution
2225 of the P8340 protease inhibitor (Sigma) by vortexing until homogenized. Fecal material
2226 was then pelleted by centrifugation at 17,000 xg for 10 min at 4°C and the supernatant
2227 collected. Fecal supernatant and serum were stored at -20°C. Mice were euthanized using
2228 isoflurane at the time points indicated and every effort was made to minimize suffering.
2229 (Further reagent details are listed in Table S1 available online at *PLOS ONE*).

2230 **5) Immunohistochemistry**

2231 Mice were gavaged with 10^8 CFU of carboxyfluorescein succinimidyl ester
2232 (CFSE) surface-labeled *S. boulardii,* and sections of small intestine were harvested one
2233 hour later, embedded in optimal cutting temperature (OCT) compound, and cryosectioned
2234 as previously described [469]. Sections were stained with VECTASHIELD anti-fade
2235 mounting media with DAPI (4′,6-diamidino-2-phenylindole).

2236 **6) ELISA**

2237 Assays for total antibody were performed by coating 96 well flat bottom
2238 MaxiSorp plates (Thermo Scientific) with unlabeled goat anti-mouse IgA and IgG
2239 (Southern Biotech) (Table S1 available online at *PLOS ONE*) diluted in

2240 carbonate/bicarbonate buffer overnight at 4°C. Alternatively, plates were coated with 10^7
2241 CFU heat-killed *S. boulardii* resuspended in carbonate/bicarbonate buffer (5.4 mM
2242 Na_2CO_3, 8.7 mM $NaHCO_3$, pH 9.6) overnight at 4°C to detect antigen specific
2243 antibodies. Plates were blocked with TBST (150 mM NaCl, 15 mM Tris HCl, 4.6 mM
2244 Tris base, 0.5% Tween 20, pH 7.6) 5% nonfat dry milk for 2 hr at room temperature (RT)
2245 prior to incubation of serially diluted samples and standards overnight at 4°C. Goat anti-
2246 mouse IgA and IgG HRP-conjugated (Southern Biotech) antibodies were incubated for
2247 1.5 hr at RT prior to addition of Super AquaBlue ELISA Substrate (eBiosciences) and
2248 reading at 405 nm. Anti-*S. cerevisiae* antibody (Abcam) and rabbit anti-goat IgG HRP-
2249 conjugated antibody (Southern Biotech) were used as positive controls for antigen
2250 specific assays. Purified mouse IgG (Invitrogen) and IgA (BD biosciences) antibodies
2251 were used as standards.

2252 **7) Flow Cytometry**

2253 Spleens, MLNs, and PPs were washed with complete Iscoves' Modified
2254 Dulbecco's Medium (cIMDM) (Iscoves' Modified Dulbecco's Medium with 10% heat
2255 inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 50
2256 µM 2-mercaptoethanol, and 1 mM sodium pyruvate, all Life Technologies except FCS
2257 from PAA laboratories) and homogenized using filtration over a 40 µm cell strainer.
2258 Samples used for analysis of CFSE-labeled yeast were resuspended in FACS buffer (1X
2259 PBS (Life Technologies), 5 mM EDTA, 2% FCS) and assayed without further staining.
2260 For experiments identifying germinal center B cells and plasma cells, homogenized cells
2261 were distributed at 10^6 cells per well in a v bottom plate and blocked with anti-CD16/32
2262 (BD biosciences). Cells were surface stained with antibody cocktails diluted in FACS

2263 buffer for 30 minutes on ice. Antibodies used include CD19 APC, Gl7 FITC, CD45R
2264 (B220) Pacific Blue, CD138 PE, all obtained from Biolegend. The Zombie NIR fixable
2265 live dead stain was also used as per manufacturer (Biolegend) protocols. Plasma cell
2266 populations were identified by Zombie^{-} CD138^{+}CD45R^{int} expression; germinal center
2267 cells were identified by Zombie^{-}CD19^{+}CD95^{+}GL7^{+} expression (Fig 6.1) [470]. For
2268 detection of anti-*S. boulardii* antibody, diluted serum and fecal samples were incubated
2269 with 10^6 whole *S. boulardii* for one hour at room temperature, followed by a 30 minute
2270 incubation with secondary goat anti-mouse IgA FITC (abcam) or donkey anti-mouse IgG
2271 PE (eBiosciences) and washes with FACS buffer. Stained cells were fixed with 2%
2272 paraformaldehyde and read on a BD LSR II flow cytometer. Analysis was conducted
2273 using FACS Diva and FlowJo software.

2274 **8) ELISPOT**

2275 Millipore Multiscreen-HA 96-well plates (Millipore #MAHA N4510) were coated
2276 with anti-mouse IgG, IgA, IgM (Rockland) diluted to 5 µg/mL in PBS and incubated
2277 overnight at 4°C. Plates were then washed with PBST (1X PBS, 0.05% Tween 20) and
2278 PBS (1X, Life Technologies) (1x PBST, 3x PBS washes) and blocked by 2 hr incubation
2279 at 37°C with cIMDM. Media was then replaced with fresh cIMDM, and counted cells
2280 from spleens, MLN, and PP were added. Plates were incubated overnight at 37°C and,
2281 following washes (4x PBS, 4x PBST), biotin-conjugated anti-mouse IgG and IgA
2282 antibodies (Southern Biotech) were added at a concentration of 0.5 µg/mL diluted in
2283 PBST 1% FCS and incubated overnight at 4°C. Plates were washed (4x PBST) before
2284 incubation with a 1:1000 dilution of HRP avidin D (Vector Laboratories) in
2285 supplemented PBS (1X PBS, 0.05% Tween 20, 1% FCS) for 1-3 hr at room temperature.

2286 After washes (3x PBST, 3x PBS), AEC substrate (0.3mg 3-amino-9-ethylcarbazole in 0.1
2287 M Na-Acetate buffer, pH 5, 0.3% hydrogen peroxide) was added and color reactions
2288 were allowed to proceed for 2-10 minutes before washing with distilled water. Plates
2289 were kept in the dark to dry until read and counted with the aid of a CTL ImmunoSpot
2290 5.1.36 analyzer.

2291 **9) RNA-sequencing**

2292 RNA extraction of MLNs from naïve and *S. boulardii*-treated C57BL/6J mice was
2293 performed using the Qiagen RNeasy mini kit with DNase treatment according to
2294 manufacturer's protocols. Sample quality analyses, library preparation, and sequencing
2295 were performed by the Huntsman Cancer Institute High Throughput Genomics Core
2296 (University of Utah). RNA integrity was confirmed using an Agilent RNA ScreenTape
2297 assay, and only high quality RNA (RIN >8.0) was submitted for further processing.
2298 Library preparation with oligo dT selection was performed using the Illumina TruSeq
2299 Stranded mRNA Sample Preparation Kit. Sequencing libraries (25 pM) were chemically
2300 denatured and applied to an Illumina HiSeq v4 single read flow cell using an Illumina
2301 cBot. Single end sequencing of 50 bp reads was performed using an Illumina HiSeq 2000
2302 according to standard protocols.

2303 A mean of 41.1 million reads per sample were acquired, with very high quality as
2304 assessed by FastQC (Babraham Institute) (Fig 6.2). Reads were mapped to the GRCm38
2305 *Mus musculus* genome (accessed via *Ensembl* [462]) using TopHat2 [471]. HT-seq [472] count
2306 was used to assign aligned reads to genes from the *Ensembl* release 82 GRCm38 genome
2307 annotation. Differential expression analysis, MA plots, and clustering were performed
2308 with DESeq2 [473]. Genes with a p-value (adjusted for multiple corrections) of 0.05 or less

2309 were considered differentially expressed. Principal component analysis was performed
2310 with two components on the log-transformed expression of the 1,000 genes with highest
2311 variance among samples using the R package psych. RNA-seq reads have been deposited
2312 to the NCBI Sequence Read Archive (SRA) under accession number SRP067985.
2313

2314 c) Results

2315 1) *S. boulardii* MYA-797 is genomically distinct from *S. cerevisiae*

2316 *S. boulardii* has therapeutic traits that are distinct from many other *S. cerevisiae*
2317 strains [323]. Furthermore, experiments with probiotic bacteria demonstrate that effects of
2318 probiotics may differ depending on the strain and even isolate [287]. To explore genomic
2319 differences of the *S. boulardii* isolate here relative to *S. cerevisiae* and other known *S.*
2320 *boulardii* isolates [464], we performed genomic sequencing of *S. boulardii* ATCC MYA-
2321 797. Hiseq Illumina sequencing of *S. boulardii* genomic DNA provided a total of
2322 105,329,454 paired end reads that were assembled using Velvet v1.2.10 into 424 total
2323 contigs, including 135 contigs of 1000 bp or more, to provide a draft genomic sequence
2324 of 11.5 Mbp with approximately 80x coverage. We identified numerous
2325 insertions/deletions (indels) and substitutions between *S. boulardii* ATCC MYA-797
2326 contigs and the sacCer3 *S. cerevisiae* reference genome (Fig 6.3A,B). More than 16,000
2327 of these changes are in exonic regions and encode amino acid substitutions. Gene
2328 ontology analysis of the genes with exonic indels and amino acid substitutions compared
2329 to *S. cerevisiae* revealed enrichment of numerous processes, including cell wall
2330 organization and assembly (Fig 6.3C, Table S2 available online at *PLOS ONE*).
2331 Alignment of sequences for genes important in cell wall formation, such as *SBE22* [474],

2332 *ALG2* [475], *LDS2* [476], and *SPR1* [477], of ATCC MYA-797 with both the sacCer3 *S.*
2333 *cerevisiae* reference genome and the previously published *S. boulardii* EDRL genome [464]
2334 reveals changes in coding regions leading to several amino acid substitutions shared by
2335 the two *S. boulardii* strains relative to *S. cerevisiae* (Fig 6.3D).

2336 **2) The *S. boulardii* cell wall is thicker relative to *S. cerevisiae* strains and**
2337 **mediates stress resistance**

2338 As sequencing of the *S. boulardii* genome revealed differences compared to *S.*
2339 *cerevisiae* in genes encoding proteins involved in cell wall formation, we compared the *S.*
2340 *boulardii* MYA-797 cell wall with two commonly used, well-characterized laboratory *S.*
2341 *cerevisiae* strains: BY4741 and W303. These strains were cryopreserved and imaged
2342 using transmission EM to visualize the cell wall (Fig 6.4). Images at low (Fig 6.4A-C,
2343 scale bar 500 nm) and high (Fig 6.4D-F, scale bar 50 nm) magnification reveal similar
2344 cell wall architecture among the studied strains. Although the major components of the
2345 yeast cell wall are integrated and not purely confined to specific lateral bands, regions of
2346 differing electron density identify cell wall layers composed primarily of these different
2347 components, namely a thin inner chitin layer, an internal β-glucan layer, and an outer
2348 mannoprotein layer [453]. Interestingly, the overall thickness of the *S. boulardii* cell wall is
2349 greater than for the two *S. cerevisiae* strains (Fig 6.4G), though this does not result from
2350 obvious increased thickness in any single cell wall layer relative to other strains. These
2351 differences in thickness and composition of the *S. boulardii* cell wall may account for
2352 some of the unique probiotic properties found for *S. boulardii* but not laboratory *S.*
2353 *cerevisiae* strains.

2354 Previous studies have found that particular cell wall components, including β-
2355 glucans, increase resistance of probiotic bacteria to pH stresses and simulated
2356 gastrointestinal conditions [478]. To examine the role of the yeast cell wall in resistance to
2357 external stresses, *S. boulardii* was treated with caspofungin and exposed to media
2358 adjusted to pH levels that would be encountered in the digestive tract. Caspofungin is an
2359 echinocandin antifungal agent that inhibits yeast (1,3)-β-D-glucan synthase [479]. Use of the
2360 phenol sulfuric acid assay, a colorimetric assay to detect total monosaccharide content,
2361 showed that, as expected, treatment with caspofungin decreased *S. boulardii* total cell
2362 wall carbohydrate content (Fig 6.5A). Interestingly, although even the highest tested
2363 concentration of caspofungin only marginally decreased growth in normal media at pH 6,
2364 growth of caspofungin-treated *S. boulardii* was significantly impaired in the presence of
2365 media adjusted to pH 4 and pH 8 relative to untreated *S. boulardii* grown at the same pH
2366 (Fig 6.5B). This data shows that the integrity of the cell wall is important for the
2367 resistance of *S. boulardii* to fluctuations in pH that would be encountered as it passes
2368 through the gastrointestinal tract.

2369 **3) Association and uptake of *S. boulardii* into small intestinal Peyer's**
2370 **patches are low frequency events**

2371 In order for any differences in cell wall composition to impact the ability of *S.*
2372 *boulardii* to deliver antigens and induce immune responses to therapeutics, the yeast must
2373 be able to contact immune cells in the intestine. To determine the degree to which *S.*
2374 *boulardii* is able to contact and adhere to antigen sampling sites, PPs from C57BL/6 mice
2375 were harvested at multiple time points after gavage with single doses of
2376 carboxyfluorescein succinimidyl ester- (CFSE) labeled *S. boulardii*. As shown in

2377 representative flow cytometry plots (Fig 6.6A), labeled yeast were clearly detected in PP
2378 samples collected one hour after gavage. Samples of luminal contents overlying collected
2379 PPs were also assayed as positive controls and demonstrate presence of yeast in the
2380 intestinal lumen at each time point after gavage, peaking at 1 hr. Quantification (Fig
2381 6.6B) of $CFSE^+$ events in PP samples at each time point shows the greatest degree of
2382 association at 1 hr.

2383 While the presence of CFSE positive events in these samples suggests association
2384 of yeast with PPs, these numbers are low relative to the initial inoculum and do not
2385 demonstrate uptake of yeast into the PPs themselves. Histological evaluation of intestinal
2386 sections from mice gavaged with CFSE-labeled *S. boulardii* further demonstrate the low
2387 frequency of intact yeast near the epithelium (Fig 6.6C). Indeed, no yeast were detected
2388 immediately adjacent to or within PPs using this approach.

2389 **4) *S. boulardii* induces marginal increases in total, but not antigen specific,**
2390 **antibody levels**

2391 The large quantities of IgA secreted into the intestinal lumen form a critical
2392 component of intestinal homeostasis and defense against invading pathogens [480]. Indeed,
2393 pathogen-specific antibody titers are the gold standard in measuring responses to vaccine,
2394 and IgA antibody titers are often used as an indicator of protection against mucosal
2395 diseases. A reported feature of the mucosal immune response to *S. boulardii* is increased
2396 total secretory IgA (sIgA) levels [132,135,137], although these studies were conducted in
2397 gnotobiotic and weanling rodent models which are known to have differences in B cell
2398 responses and antibody levels relative to specific-pathogen-free (SPF) mice [208]. To
2399 determine the nature of *S. boulardii*-induced antibody production in adult SPF mice and

2400 to determine if prolonged exposure to *S. boulardii* further increases antibody levels over
2401 time, C57BL/6 mice were orally gavaged with 10^8 CFU *S. boulardii* or control vehicle
2402 daily for 7, 14, or 28 days, and sera and fecal samples were collected to determine
2403 antibody levels.

2404 As expected, total fecal IgA levels as determined by ELISA increase in *S.*
2405 *boulardii*-treated mice relative to naïve mice until day 28, although this did not reach
2406 statistical significance (Fig 6.7A). *S. boulardii*-treated mice also showed increases in total
2407 serum IgG and IgA, suggesting that oral gavage with *S. boulardii* induces some degree of
2408 systemic immune effects, although limited. *Ex vivo* incubation of *S. boulardii* with serum
2409 and fecal supernatant collected from naïve and *S. boulardii*-gavaged mice furthermore
2410 enabled detection of *S. boulardii*-reactive antibody by flow cytometry (Fig 6.7B, C). The
2411 percent of *S. boulardii* opsonized with antibody did not increase when cells were
2412 incubated with samples from *S. boulardii*-gavaged mice relative to samples from control
2413 mice, indicating no increase in anti-*S. boulardii* antibody levels in treated mice. Analysis
2414 by ELISA also demonstrated no detectable levels of *S. boulardii*-reactive antibody in
2415 either group (Fig 6.8). These findings indicate that although *S. boulardii* induces both a
2416 local and systemic increase in total antibody, this response requires numerous doses to
2417 reach significance and does not induce antibodies reactive against yeast antigens
2418 themselves.

2419 **5) *S. boulardii* induces limited changes in numbers of germinal center B cells**
2420 **and plasma cells**

2421 To further investigate the nature of B cell responses to *S. boulardii*, mice were
2422 gavaged with daily doses of vehicle or 10^8 CFU *S. boulardii* for 28 days. Peyer's patches

(PP), mesenteric lymph nodes (MLN) and spleens were assayed for numbers of germinal center B cells ($C19^{+}Gl7^{+}CD95^{+}$) (Fig 6.9A) and plasma cells ($CD138^{+}B22^{int}$) (Fig 6.9B) by flow cytometry (gating strategy shown in Fig 6.1). Quantification indicated no significant differences in the number of germinal center B cells or plasma cell in the PPs, MLNs, or spleens of *S. boulardii*-treated versus naïve mice. Total cell numbers of each tissue were not significantly different between groups, and cell percentages reflected similar patterns seen by cell number (Fig 6.10). Thus there are only minimal differences induced in B cell populations by *S. boulardii* in the healthy immune system.

6) *S. boulardii* induces trends toward increased numbers of antibody secreting cells

To further analyze antibody responses to *S. boulardii*, ELISPOT analysis of PPs, MLNs, and spleens harvested from mice after 28 days of gavage was used to enumerate antibody secreting cells in these tissues. Although IgA and IgG secreting cells showed consistent trends toward increased numbers in *S. boulardii*-treated mice, none of these differences reached statistical significance (Fig 6.9C).

7) *S. boulardii* induces minimal changes in MLN gene expression

To quantify gene expression changes in other immune pathways induced by *S. boulardii* treatment, we isolated RNA from whole MLNs of mice gavaged daily with 10^{8} CFU of *S. boulardii* or PBS. RNA from two mice in each group was sequenced, for a total of 164 million reads. Reads were aligned to the GRCm38 mouse genome and gene expression changes were calculated using DESeq2 (http://www.genomebiology.com/content/15/12/550). 19,601 total genes were identified with 5 or more normalized counts.

2446 Overall, expression of very few genes changed significantly with *S. boulardii*
2447 treatment versus vehicle (Fig 6.11A, Table S3 available online at *PLOS ONE*); only
2448 fourteen genes were identified as differentially expressed (p-value adjusted for multiple
2449 comparisons < 0.05) between the two groups. Both principal component analysis (Fig
2450 6.11B) and clustering analysis (Fig 6.11C) demonstrate that gene expression differences
2451 detected are driven by differences between individual mice rather than any differences
2452 induced by *S. boulardii* treatment.
2453

2454 **d) Discussion and Conclusions**

2455 Probiotic organisms provide clinical benefits in the context of numerous
2456 infectious and inflammatory gastrointestinal disorders. However, current prophylactic use
2457 of these probiotics [481] as well as their potential application for delivering therapeutics to
2458 the gastrointestinal tract [331,332,408] necessitates an understanding of how these organisms
2459 interact with the uninflamed, healthy mucosa. Probiotic organisms may exert beneficial
2460 effects through modulation of the immune system, yet the immunogenicity of probiotics
2461 within the healthy intestine is not well described. In this study, we have assessed intrinsic
2462 properties and immunomodulatory interactions of the probiotic yeast *S. boulardii* in the
2463 healthy intestine.

2464 Previous studies have reported that *S. boulardii* administered to mice has
2465 immunomodulatory effects such as induction of antibodies [132,135,137]. In our hands, *S.*
2466 *boulardii* does not invoke a significant immune response in the context of the healthy
2467 gastrointestinal tract. However, we have identified a number of genomic and phenotypic
2468 differences between *S. boulardii* and non-probiotic *S. cerevisiae* that may explain some

2469 of the probiotic effects of *S. boulardii* in disease states. For example, cell wall thickness
2470 of *S. boulardii* was noticeably greater compared to *S. cerevisiae* (Fig 6.4), and this is
2471 consistent with genomic differences in genes encoding proteins involved in cell wall
2472 function (Fig 6.3).

2473 Although no *S. boulardii*-reactive antibody was detectable even after multiple
2474 doses of *S. boulardii*, we did observe statistically significant, albeit marginal, increases in
2475 serum IgA and IgG (Figs 6.7 and 6.8). These data are consistent with previous studies
2476 showing no effect on the number of antibody-producing B cells in human peripheral
2477 blood mononuclear cells (PBMCs) upon consumption of *S. boulardii* (31 and 32). Our
2478 studies in mice enabled a more detailed, *in vivo* analysis of localized immune responses
2479 in the gastrointestinal tract. However, we did not detect any significant changes in
2480 antibody levels or B cell populations in the mucosa (Figs 6.7-6.9). An extended analysis
2481 to evaluate potential effects of *S. boulardii* on other immune cell pathways by performing
2482 RNA-seq of the mesenteric lymph nodes further confirmed the minimal impact of *S.*
2483 *boulardii* on the local mucosal immune response (Fig 6.11). The minimal number of gene
2484 expression changes detected in this analysis suggest that administration of *S. boulardii* in
2485 the context of the healthy mucosa does not induce an inflammatory immune response and
2486 has little effect in potentiating B and T cell responses.

2487 Results of the present study are consistent with a recent report that the probiotic
2488 bacterium *Lactobacillus rhamnosus* GG influences the T helper cell balance in Crohn's
2489 disease patients but not in healthy control patients [448]. These findings led to the
2490 suggestion that effects of probiotics are limited in the healthy intestine, possibly due in
2491 part to restricted contact between probiotics and immune cells. However, in the inflamed

intestine, disruption of barrier integrity, microbiota changes, and recruitment of inflammatory cells likely all increase the frequency of encounters between probiotics and immune cells. Our data strongly suggest that in the healthy adult mouse intestine the majority of *S. boulardii* do not contact the gastrointestinal epithelium, including the Peyer's patches of the small intestine (Fig 6.6). If uptake of whole intact yeast is indeed a low frequency event, use of *S. boulardii* as a vaccine delivery vector may require optimization to increase efficiency of antigen delivery to immune cells underlying the epithelium.

Several approaches may permit increased contact of *S. boulardii* with the epithelium and improve therapeutic delivery. Studies examining use of probiotic bacteria as delivery vehicles have proposed heterologous expression of M cell ligands to increase contact and uptake of probiotics by these antigen sampling cells on Peyer's patches [482,483]. It is known that particle uptake by M cells is size-dependent [444], and in the case of *S. boulardii* (3-10 μm average diameter) uptake of whole yeast may be restricted. Inducing vaccine antigen secretion such as through use of the *S. cerevisiae* alpha mating factor prepro leader sequence may thus be needed to enable uptake by antigen sampling cells [331]. This approach in combination with expression under the control of promoters that are activated in response to the alkaline or low oxygen conditions of the small intestine [484], may prove beneficial for future applications using *S. boulardii* to deliver heterologous protein to the intestine. These features have already been explored for a strain of probiotic bacteria [485], and the genomic sequences of multiple *S. boulardii* isolates now available will facilitate identification and cloning of promoters for this probiotic yeast.

The limited immunomodulatory effects of *S. boulardii* in the healthy intestine have several implications for its use as a vaccine delivery vector as well as a prophylactic agent. Prophylactic efficacy may be due to local effects of *S. boulardii* within the intestine such as by contributing to maintenance of a normal microbiota [274] or affecting epithelial integrity, such as through trophic effects as have been previously described [382,486]. These beneficial attributes may help buffer the intestine against pathogenic or inflammatory challenge; however, results of the present study suggest that protective effects of *S. boulardii* in the healthy intestine are not immune-mediated. As a vaccine delivery vector, the limited immunomodulation of *S. boulardii* in the healthy intestine may allow for applications in a wide range of diseases. Such a vector could potentially be used to deliver vaccine antigen without inducing damaging inflammatory responses that would lead to rapid yeast clearance and prevent adequate therapeutic delivery. Furthermore, the lack of T helper cell polarization in response to *S. boulardii* could allow for use of specific adjuvants in combination with *S. boulardii* to arm particular immune effector mechanisms in a manner tailored to the pathogen of interest.

In summary, we present an investigation into both intrinsic and immunological properties of the probiotic yeast *S. boulardii* in the healthy intestine. We show that delivery of *S. boulardii* to mice is relatively benign with respect to the induction of a mucosal immune response, suggesting that *S. boulardii* may exert reported beneficial effects in the healthy gastrointestinal tract by mechanisms that are not based on immune system modulation. These findings inform future work using *S. boulardii* in the healthy intestine and provide rationale for better optimization and testing of *S. boulardii* as a vaccine delivery vector.

e) Acknowledgements

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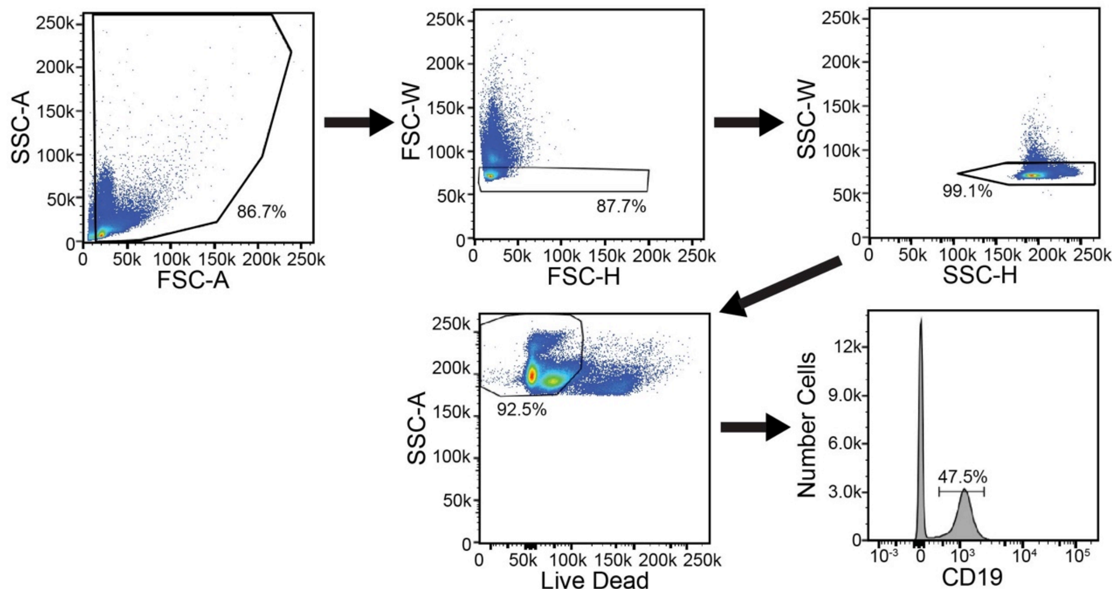
2549 **f) Figures and Tables**

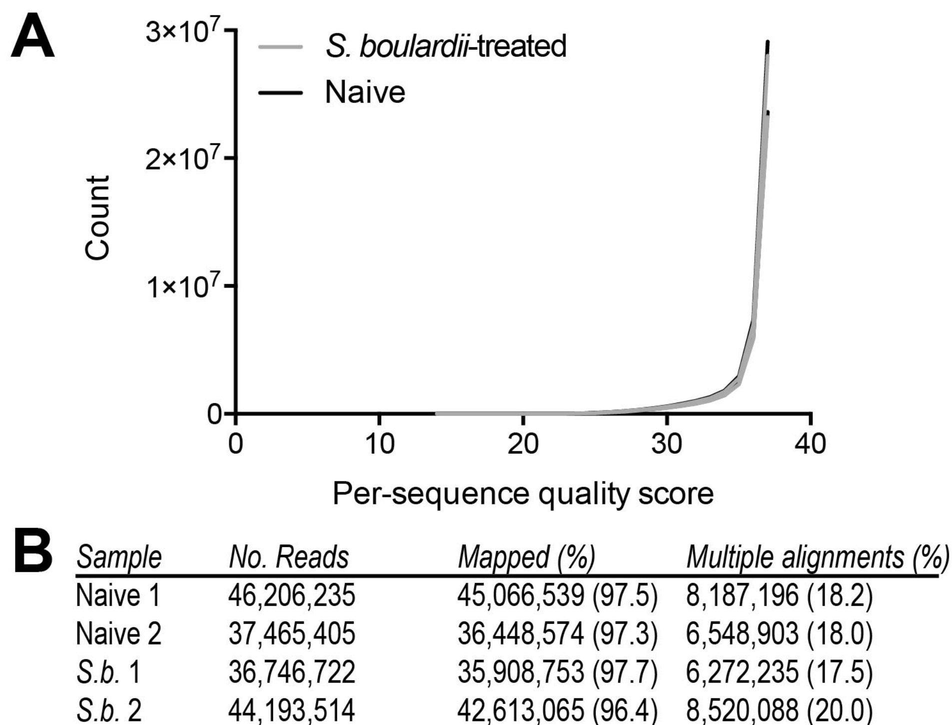


2550

2551 **Fig 6.1. Gating strategy for B cell flow cytometry panel**

2552 Cell were gated first on lymphocytes, then single cells by FSC-H FSC-W and
2553 SSC-H SSC-W gates, and then Zombie NIR negative populations to determine live cells
2554 from which plasma cells were then gated. To determine germinal center B cells, live cells
2555 were further gated to identify the $CD19^+$ population.

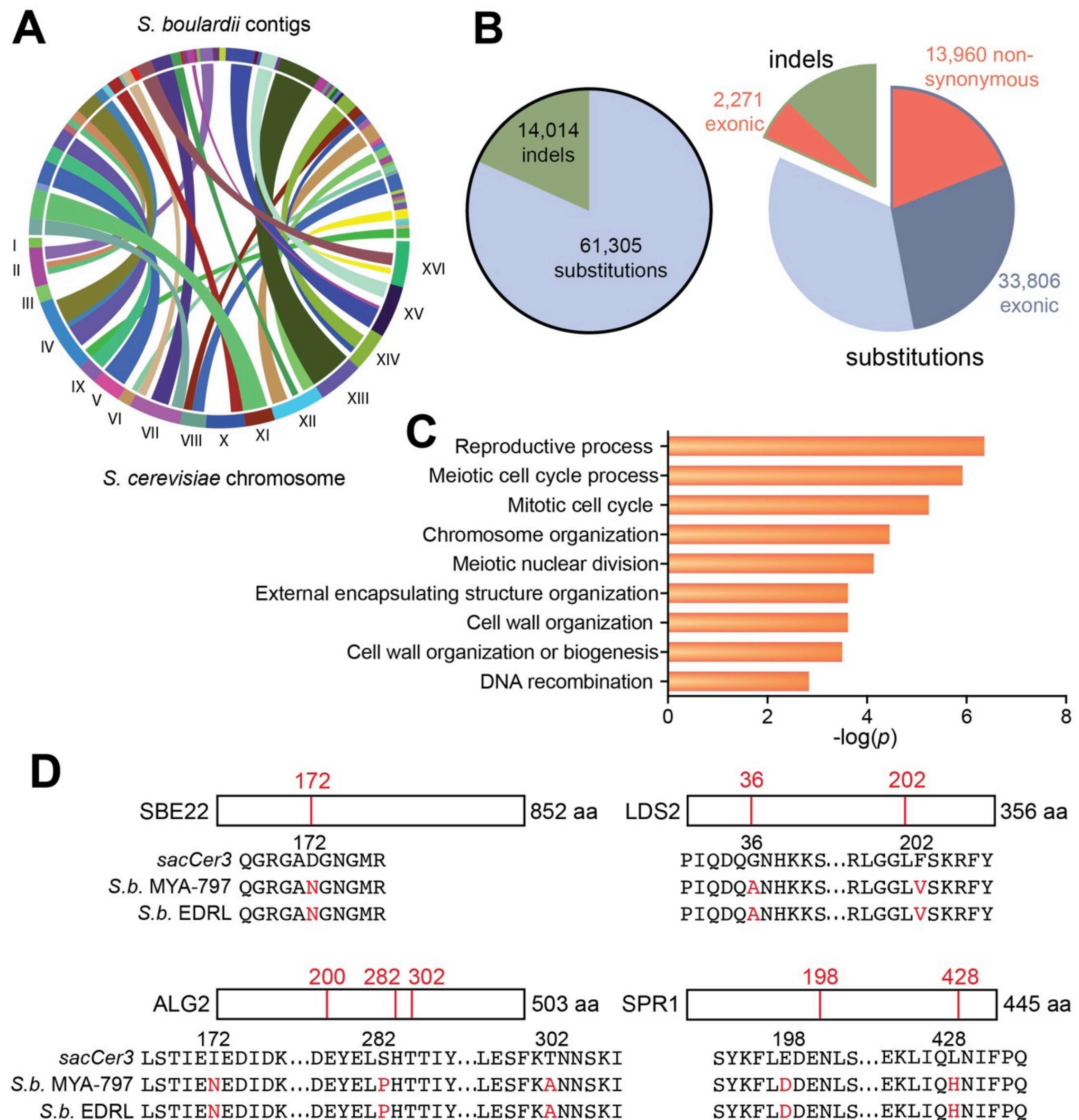
2556



Sample	*No. Reads*	*Mapped (%)*	*Multiple alignments (%)*
Naive 1	46,206,235	45,066,539 (97.5)	8,187,196 (18.2)
Naive 2	37,465,405	36,448,574 (97.3)	6,548,903 (18.0)
S.b. 1	36,746,722	35,908,753 (97.7)	6,272,235 (17.5)
S.b. 2	44,193,514	42,613,065 (96.4)	8,520,088 (20.0)

Fig 6.2. RNA-seq read quality and mapping statistics

(A) Per-sequence quality score (as determined by FastQC) of the four sequenced samples. (B) Bowtie2 alignment summary for each sample.



2562

2563 **Fig 6.3 Sequencing of the *S. boulardii* genome reveals changes in genes involved in**

2564 **cell wall organization**

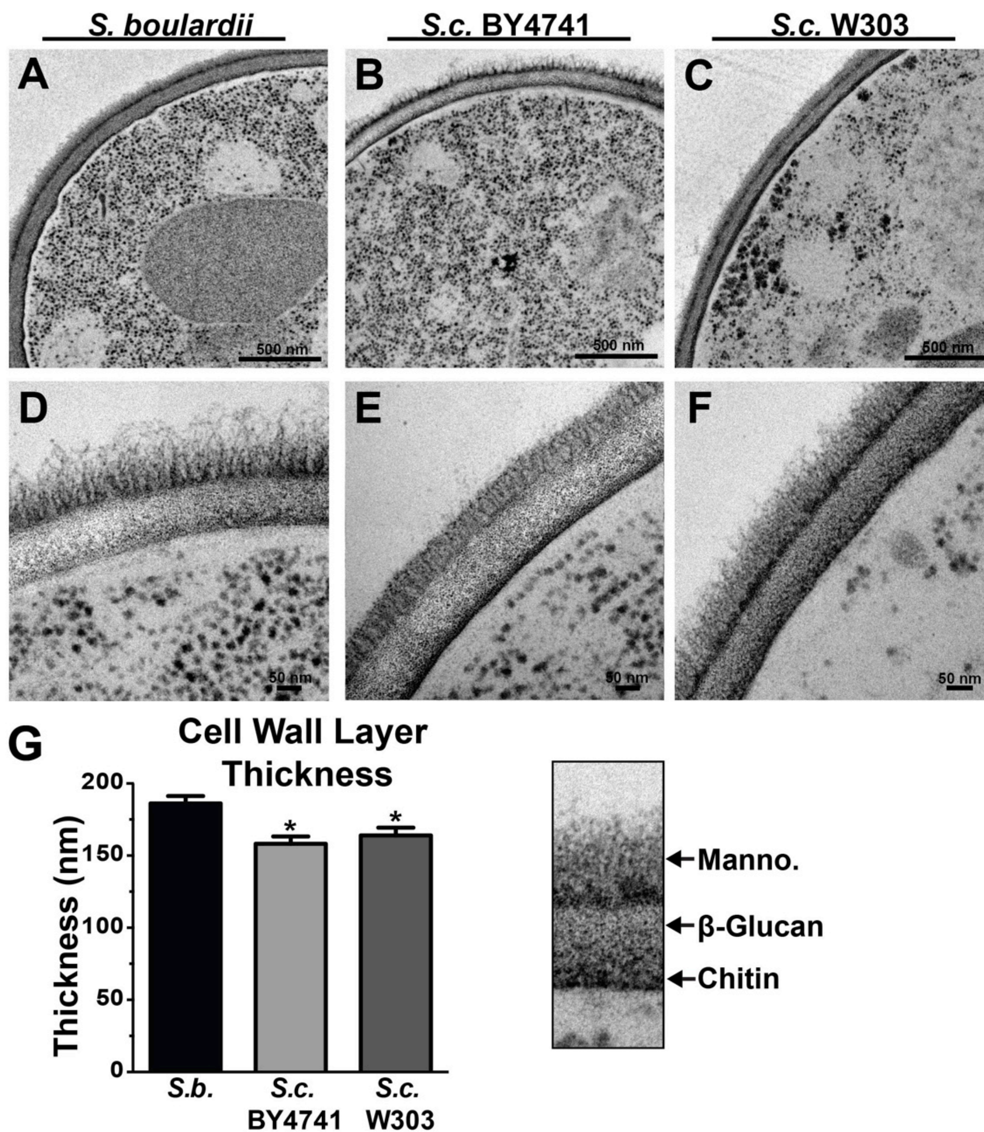
2565 (A) The *S. boulardii* ATCC MYA-797 genome was sequenced, yielding an 11.5 Mbp

2566 draft genome with 135 contigs of 1000 bp or more. Shown is a circle plot depicting

2567 synteny between the draft genome contigs and the *S. cerevisiae* sacCer3 reference

2568 genome. (B) Summary of sequence differences between the *S. boulardii* draft genome

2569 reported here and the *S. cerevisiae* reference genome. (C) Gene ontology analysis reveals
2570 that differences between *S. boulardii* and *S. cerevisiae* coding regions occur in genes
2571 critical for cell wall formation. Selected ontology terms and their Holm-Bonferroni *p*-
2572 values are shown. (D) Examples of the amino acid substitutions in the coding regions of
2573 *SBE22* [474], *ALG2* [475], *LDS2* [476], and *SPR1* [477], which all play important roles in cell wall
2574 formation.
2575



2576

2577 **Fig 6.4 The cell wall of *S. boulardii* is thicker than in *S. cerevisiae* strains**

2578 *S. boulardii* (A, D) and *S. cerevisiae* BY4741 (B,E) and W303 (C, F) were cryopreserved

2579 and imaged via transmission electron microscopy. Scale bars denote 500 nm (A-C) and

2580 50 nm (D-F). (G) Quantification of total cell wall thickness for each strain was calculated

2581 taking the average of 23 cells per strain. Error bars depict the standard error of the mean

2582 (SEM), * $p < 0.05$ relative to *S. boulardii*, Kruskal-Wallis with Dunn's multiple

2583 comparison test.

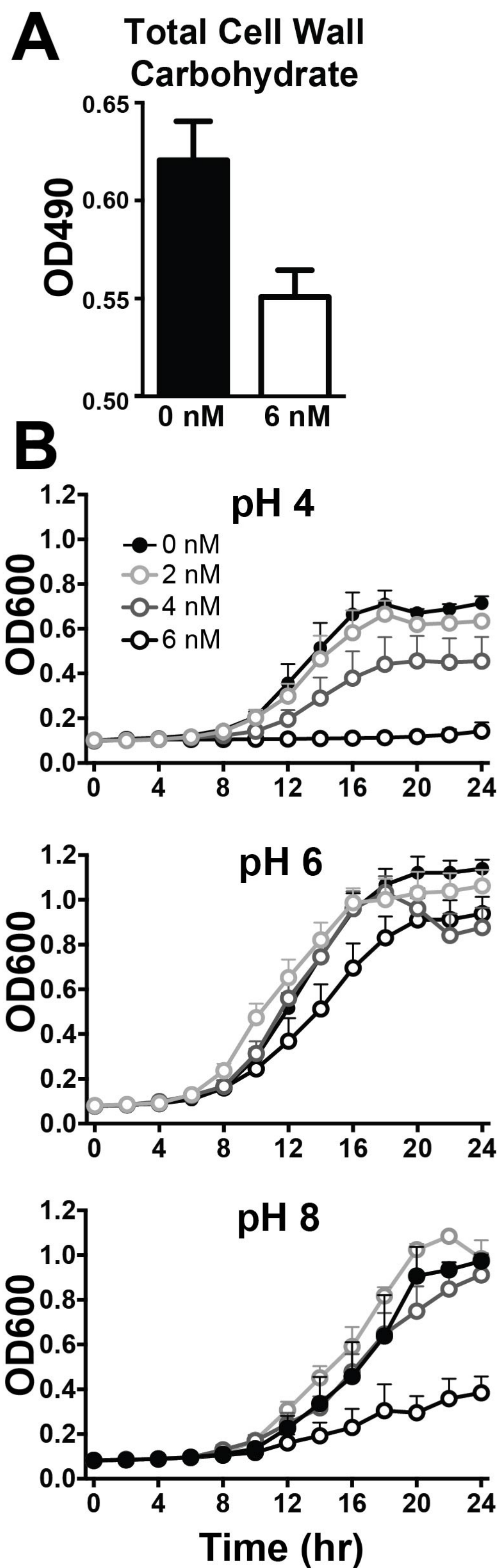


Fig 6.5 Caspofungin treatment alters *S. boulardii* cell wall structure and decreases resistance to pH

(A) To assess cell wall integrity,10^9 yeast grown to saturation in either normal YPD media (0 nM) or media containing 6 nM caspofungin (6 nM) were tested using the phenol sulfuric acid assay, with higher OD_{490} readings indicating greater overall cell wall carbohydrate content. (B) Growth of *S. boulardii* in the presence of varying concentrations of caspofungin (0-6 nM) in normal media at pH 6 or media adjusted to pH 4 and pH 8 was measured by optical density (OD_{600}) readings of cultures at the indicated times.

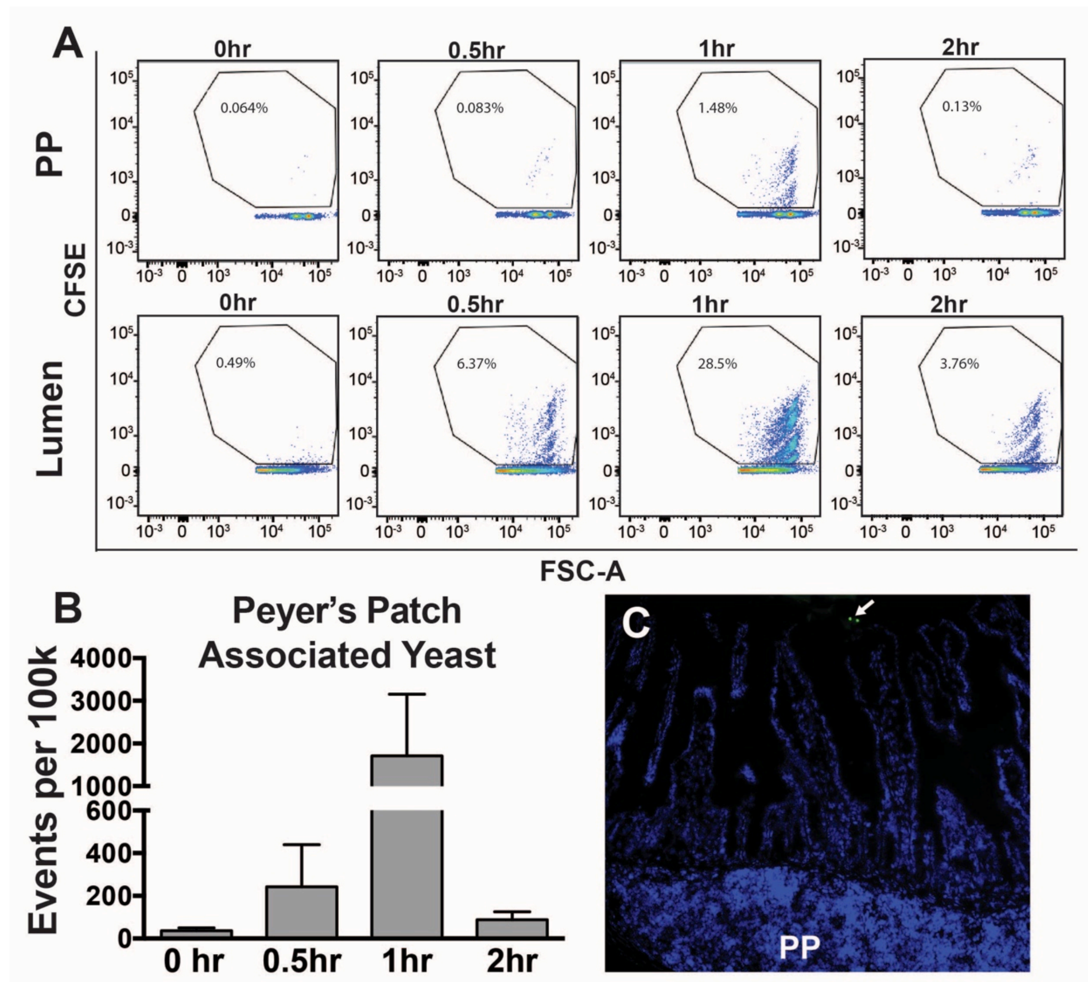
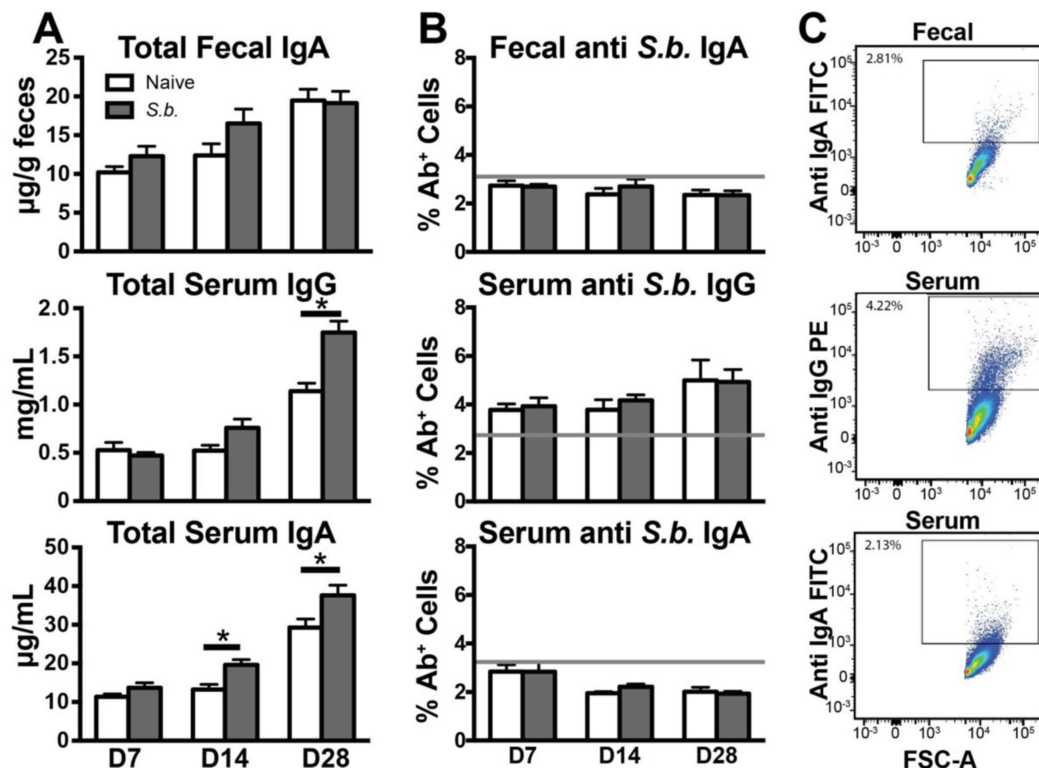


Fig 6.6 *In vivo* contact of *S. boulardii* with murine Peyer's patches is limited

(A) C57BL/6 mice were gavaged with 10^8 CFSE surface-labeled *S. boulardii*. Peyer's patches (PP) and overlying luminal contents (Lumen) were collected 0, 0.5, 1, and 2 hr post gavage and analyzed by flow cytometry to detect $CFSE^+$ events, as shown in representative flow plots. (B) Quantification of $CFSE^+$ events per 100,000 cells in PP samples shows greatest association of yeast with these immune tissues at one hour post gavage (n = 4 PP per mouse in each of two independent experiments; error bars depict the standard error of the mean). (C) Immunohistochemistry showing CFSE-labeled *S. boulardii* (arrow) is largely excluded from contact with intestinal epithelial cells (DAPI) and a small intestinal Peyer's patch (PP).



2613

2614 **Fig 6.7 *S. boulardii* induces increased total but not antigen specific fecal and serum**

2615 **antibody levels**

2616 (A) Total fecal IgA, serum IgG, and serum IgA levels were determined by ELISA using

2617 samples from mice gavaged with vehicle (white bars) or 10^8 CFU *S. boulardii* (gray bars)

2618 daily for 7, 14, or 28 days (B) Percentage of *S. boulardii* cells positive for IgA or IgG

2619 after incubation with serum or fecal samples collected from naïve mice (white bars) or

2620 mice gavaged daily with *S. boulardii* (gray bars) for 7, 14, or 28 days, with gray lines

2621 showing the average percentage of stained cells in control samples incubated with

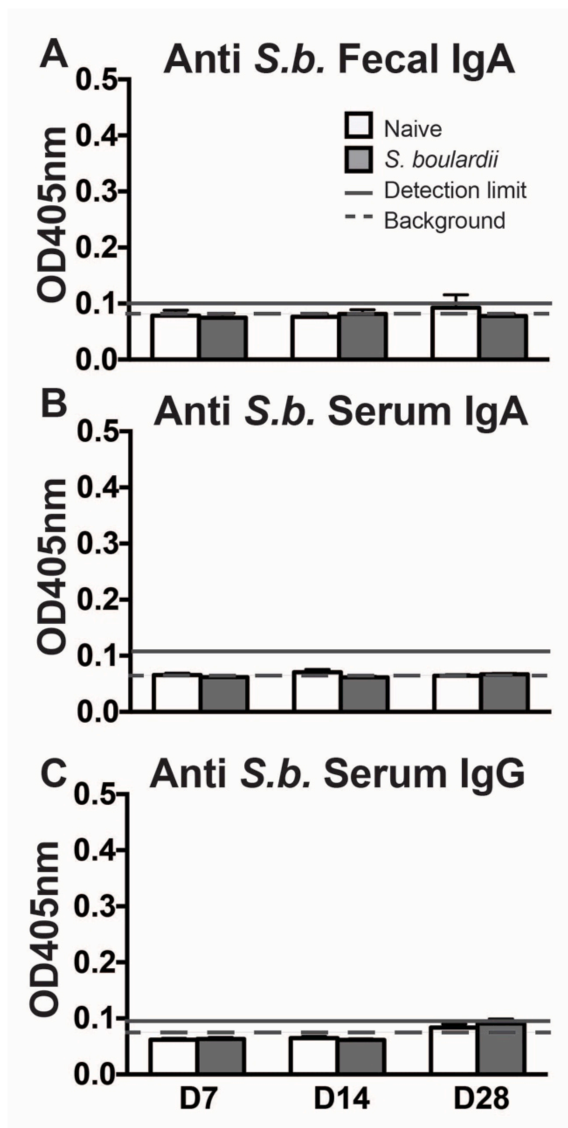
2622 secondary antibody only. (C) Representative flow plots depicting the percent of total *S.*

2623 *boulardii* cells positive for IgA or IgG after incubation with either serum or fecal

2624 supernatant. (n = 5 mice per group in each of two independent experiments per time

2625 point, with error bars showing the standard error of the mean (SEM), *$p<0.05$, ordinary

2626 two-way ANOVA, Sidak multiple comparison test).



2627

2628 **Fig 6.8. Anti-*S. boulardii* antibody levels as determined by ELISA are below**

2629 **detectable limits**

2630 Plates coated with heat-killed *S. boulardii* were used in ELISA to determine *S. boulardii*

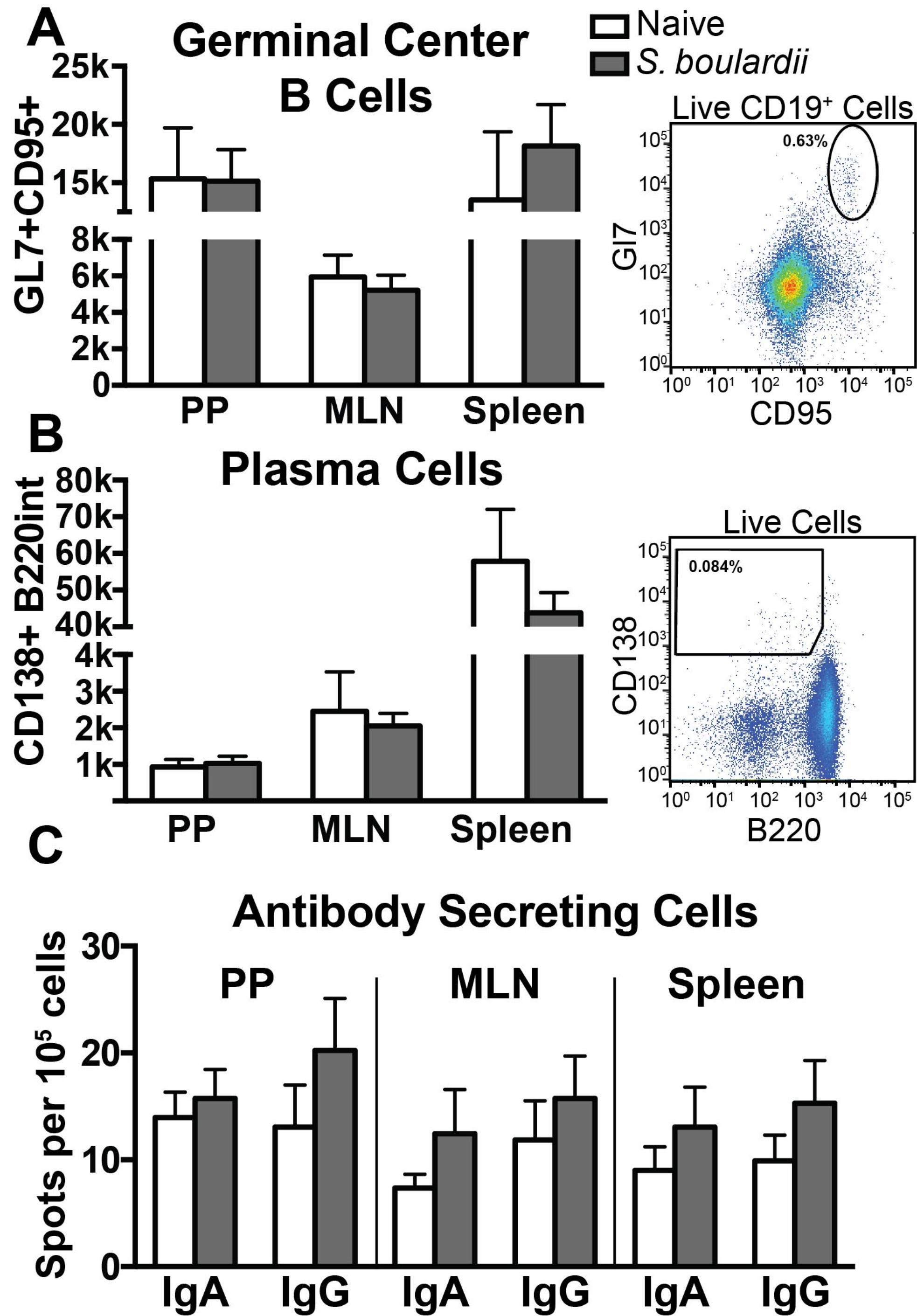
2631 specific antibody levels in the feces (A) and serum (B,C) of naïve (white bars) and *S.*

2632 *boulardii*-treated (gray bars) mice. No antigen specific antibody levels were above

2633 detectable limits at days 7, 14, or 28. Limit of detection (solid line) was determined using

2634 a control anti-*Saccharomyces cerevisiae* antibody, and background level (dashed line)

2635 was determined using the OD_{405} readings of blank wells.



2636

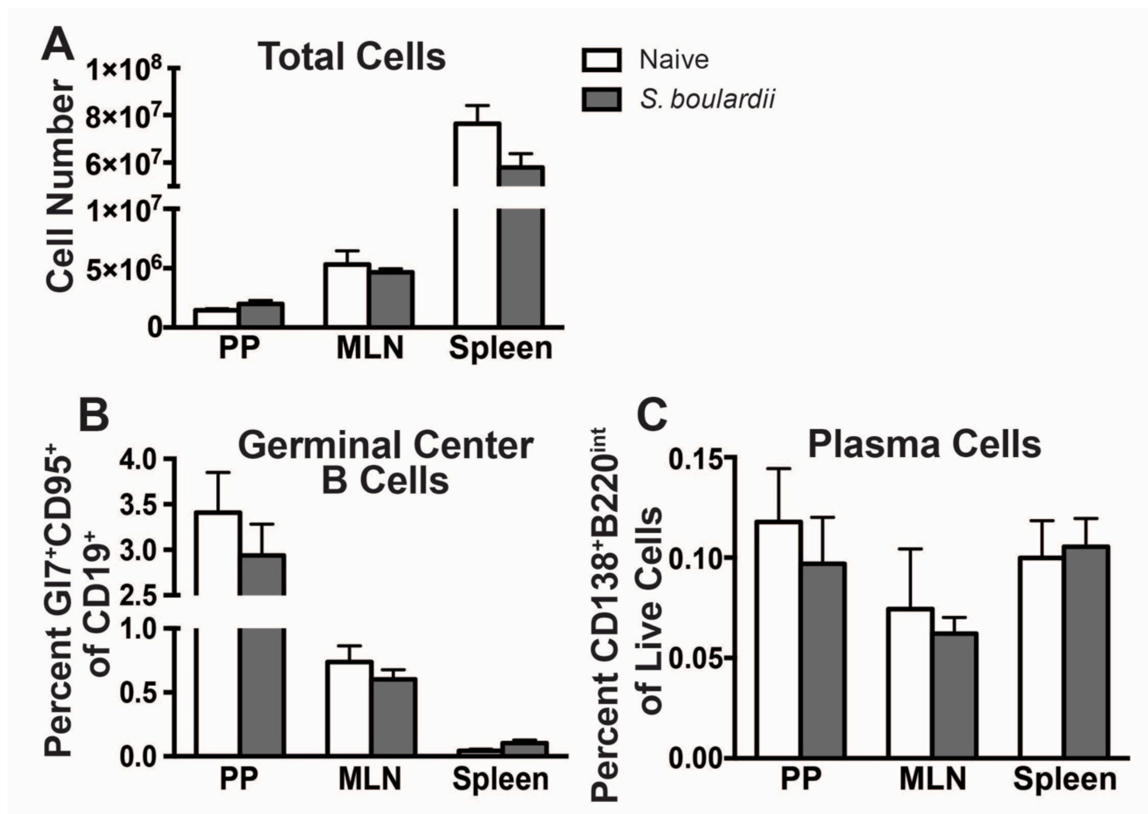
2637 **Fig 6.9. *S. boulardii* gavaged mice show only marginal differences in B lineage cell**

2638 **populations**

2639 Peyer's patches (PP), mesenteric lymph nodes (MLNs), and spleens were collected from

2640 mice gavaged daily with vehicle (white bars) or 10^8 CFU *S. boulardii* (gray bars) for 28

2641 days and analyzed by flow cytometry and ELISPOT to analyze *S. boulardii*-induced
2642 changes in B cell lineage cells. Representative flow plots and quantification of total
2643 numbers of germinal center B cells ($CD19^{+}GL7^{+}CD95^{+}$) (A) and plasma cells
2644 ($CD138^{+}B220^{int}$) (B) show no statistically significant differences in these populations (n
2645 = 5 mice per group in each of two independent experiments). (C) Spots per $5x10^{5}$ cells
2646 indicate the number of IgA or IgG secreting cells in each tissue, as determined by
2647 ELISPOT. Error bars depict SEM.
2648

2649 

2650 **Fig 6.10. Cell numbers and percentages of plasma cells and germinal center B cells**
2651 **are not significantly different**

2652 (A) Numbers of total live cells as determined by hemocytometer counts with trypan blue
2653 staining show no difference in the size of PPs, MLNs, or spleens of *S. boulardii*-treated
2654 (gray bars) and naïve (white bars) mice. Percentages of germinal center B cells (B) and
2655 plasma cells (C) in each tissue as determined by flow cytometry are also not statistically
2656 different.

2657

2658

2659

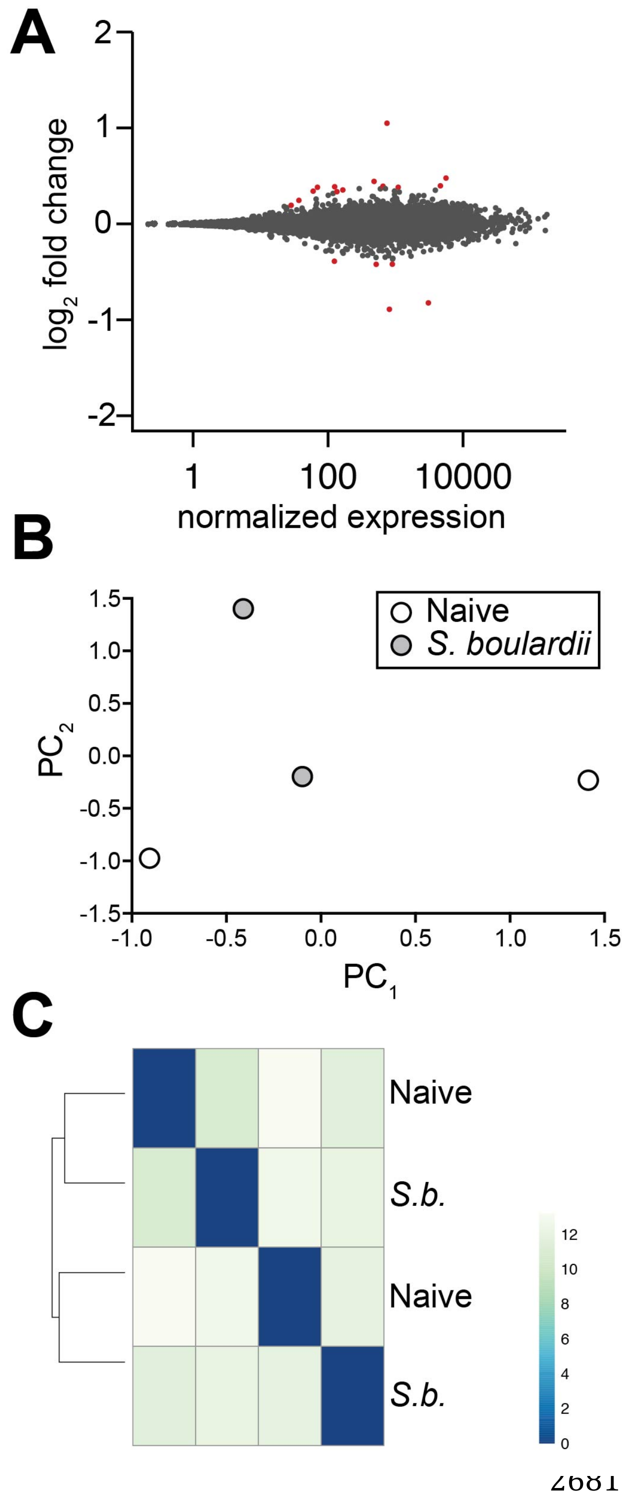


Fig 6.11. RNA-sequencing of MLNs reveals few differences in gene expression between *S. boulardii*-treated and naïve mice. (A) MA plot depicting log scale average gene expression versus fold change of gene expression in *S. boulardii*-treated versus naïve mice on the x and y axes, respectively. Differentially expressed genes are shown in red. (B) Principal component analysis and (C) sample-to-sample distance clustering of naïve and *S. boulardii*-treated samples demonstrate no large effect of *S. boulardii* on gene expression in mouse MLNs.

2681

7) Functional Heterologous Protein Expression by Genetically Engineered Probiotic Yeast *Saccharomyces boulardii*

The following is adapted from the article by Lauren E. Hudson, Milo B. Fasken, Courtney D. McDermott, Shonna M. McBride, Emily G. Kuiper, David B. Guiliano, Anita H. Corbett, and Tracey J. Lamb published in 2014 in *PLOS ONE* (doi:10.1371/journal.pone.0112660).

Figures of Ura3 structure and mutations in *S. boulardii* M1-M3 were generated by Emily Kuiper.

a) Introduction

Saccharomyces cerevisiae subspecies *boulardii* is a generally recognized as safe (GRAS) yeast strain classified as a subspecies of the well characterized laboratory yeast *S. cerevisiae* [319,320]. *S. boulardii* is currently used as a probiotic to treat antibiotic-induced diarrhea in children and adults, recurrent *Clostridium difficile* infections, inflammatory bowel disease, and other gastrointestinal disorders [312,313]. The exact mechanisms by which *S. boulardii* mediates these protective effects are not fully understood. However, administration of *S. boulardii* in animal models has been shown to increase secretory IgA, interleukin 10 (IL-10), and IL-10 induced T regulatory cells [136,487] as well as to preserve intestinal epithelial integrity in colitis models [138,329,488] and to degrade specific pathogen toxins [129,489].

Key features of *S. boulardii* have raised the interesting prospect of using this probiotic yeast not only as a preparation of wild type cells for the treatment of

2705 gastrointestinal disorders, but also as a vehicle for drug synthesis and delivery to the
2706 intestine. First, targeted delivery of drug to the gastrointestinal tract could permit lower
2707 drug doses relative to systemic administration as well as facilitate more direct interactions
2708 with the mucosal immune system. Second, genetically modified yeast would be a less
2709 expensive alternative to many proposed delivery mechanisms, such as nanoparticles and
2710 liposomes, as yeast can be economically produced on a large, industrial scale. Indeed, *S.*
2711 *cerevisiae* is already used to produce such compounds as insulin, hepatitis B surface
2712 antigen, granulocyte macrophage colony stimulating factor (GM-CSF), and platelet
2713 derived growth factor (reviewed in [334]).

2714 *S. boulardii* also has several advantages relative to other live microorganisms
2715 proposed as drug delivery vehicles. As a eukaryotic organism capable of expressing
2716 complex, glycosylated antigens, *S. boulardii* can potentially express a much wider array
2717 of compounds than probiotic bacteria. Also, *S. boulardii* shows increased resistance to
2718 higher temperatures and low pH relative to conventional laboratory strains of *S.*
2719 *cerevisiae* [309,310], which could translate to an increased ability of *S. boulardii* to survive
2720 transit through the intestine. Furthermore, *S. boulardii* is not a natural colonizer of the
2721 gastrointestinal tract in humans or mice [324–326,490], which would allow for accurate drug
2722 dosing given reliable clearance of *S. boulardii* from the intestine.

2723 Although transformation of DNA into *S. boulardii* has been reported to be less
2724 efficient than transformation of DNA into *S. cerevisiae* [330], various methods of
2725 transformation have recently been evaluated in *S. boulardii* [333]. Several studies have
2726 reported successful transformation of DNA and production of recombinant protein in *S.*
2727 *boulardii* [309,316,330–332]; however, feasibility of this application is currently limited because

prototrophic, wild type (WT) *S. boulardii* can be transformed and selected only with antibiotic resistance markers. Clinical use of these transformed yeast on a large scale would thus carry risk of transferring antibiotic resistance markers to the microbiota. A common alternative to antibiotic selection of transformed yeast is the use of auxotrophic mutants. Auxotrophic yeast lack enzymes critical for the synthesis of essential amino acids or pyrimidines and can grow in selective media only if they are transformed with a plasmid encoding the required enzyme. Unfortunately, the only existing *S. boulardii* auxotroph is unavailable for use in the United States [332]. Thus there remains a need to generate an auxotrophic strain of *S. boulardii* that can be easily manipulated without the use of antibiotic resistance markers. This auxotrophic strain would also need to produce recombinant protein during transit through the gut despite the harsh digestive conditions and lack of selective pressure. Such an auxotrophic strain would make *S. boulardii* a much safer and more efficient vehicle to express and deliver recombinant proteins to treat gastrointestinal disorders.

To develop a strain of *S. boulardii* that can be transformed without antibiotic selection markers, we used a UV mutagenesis approach and selected for auxotrophic mutants that lack a functional orotidine 5'-phosphate decarboxylase (Ura3), encoded by the *URA3* gene. The Ura3 enzyme decarboxylates orotidine monophosphate (OMP) to form uridine monophosphate (UMP) in the *de novo* synthesis pathway of pyrimidines. The $ura3^-$ auxotrophic yeast generated are unable to grow on media lacking uracil, allowing for positive selection of $ura3^-$ mutant yeast transformed with a *URA3* plasmid on media lacking uracil. In addition, Ura3 converts the compound 5-fluoroorotic acid (5-

2750 FOA) to the toxin 5-fluorouracil, inducing cellular death of *URA3*$^{+}$ yeast plated on media
2751 containing 5-FOA and allowing for easy identification of *ura3*$^{-}$ colonies.

2752 Here we employed UV mutagenesis and 5-FOA screening to generate three *S.*
2753 *boulardii ura3*$^{-}$ auxotrophic mutants. These mutants can be transformed and selected
2754 without the use of antibiotics. Furthermore, these mutants maintain the resistance to bile
2755 acid and low pH that is characteristic of WT *S. boulardii* and are taken up into immune
2756 tissues of the murine gastrointestinal tract at a frequency similar to that of WT *S.*
2757 *boulardii*. These mutant yeast also continue to express functional recombinant protein
2758 after passage through the intestine and uptake into immune tissues. In sum, we have
2759 developed *S. boulardii* strains that could be adapted for use in the synthesis and delivery
2760 of drug to the gastrointestinal tract.
2761

2762 **b) Materials and Methods**

2763 **1) Screening of UV Irradiated Cells**

2764 WT *S. boulardii* was prepared and irradiated as for generation of survival curves
2765 with *S. cerevisiae rad1* used as a control. Cells were given doses of UV irradiation
2766 corresponding to approximately 50% WT *S. boulardii* survival (20,000-22,500 μJ) and
2767 plated onto media containing 5-fluoroorotic acid (5-FOA) to select for cells lacking a
2768 functional *URA3* gene [491,492]. Colonies resistant to 5-FOA were screened by multiple
2769 restreaking onto new plates containing 5-FOA and plates lacking uracil as well as by
2770 assessing growth in liquid media lacking uracil.

2771 **2) Confirmation of URA3 mutations**

2772 The primers URA3_Fwd
2773 (CCTGCAGGAAACGAAGATAAATCATGTCGAAAGCTACATA) and URA3_Rev
2774 (CATTTACTTATAATACAGTTTTTAGTTTTGCTGGCCGCA) were used to PCR
2775 amplify the 804 bp *URA3* coding region of the *S. boulardii* mutants. PCR products were
2776 purified using the PCR Purification Kit (Qiagen) and submitted to Beckman Coulter
2777 Genomics for sequencing.

2778 **3) pH and Bile Acid Testing**

2779 WT *S. boulardii*; *S. boulardii ura3*$^-$ Mutants 1, 2, and 3; and *S. cerevisiae*
2780 laboratory haploid (W303), diploid (YH990), and WT haploid (Rm11-1a) strains were
2781 grown overnight in YPD. Cells ($5x10^7$) were then resuspended in 500 μL YPD
2782 (approximately pH6); YPD adjusted to pH 2, 4, or 8 via addition of either 12 N HCL or
2783 NaOH; complete media containing 0.3% OxGall (US Biologicals); or media lacking
2784 uracil. For each dilution, 100 μL was aliquoted in duplicate in 96 well plates (10^7 cells
2785 per well), and optical density 600 (OD_{600}) readings were taken over 24 hour incubation at
2786 37°C using a Bio Tek Instruments ELx 808 Ultra Microplate Reader to assess growth.

2787 **4) Anaerobic Testing**

2788 WT *S. boulardii*; *S. boulardii ura3*$^-$ Mutants 1, 2, and 3; and *S. cerevisiae*
2789 laboratory haploid (W303), diploid (YH990), and WT haploid (Rm11-1a) strains were
2790 grown overnight in YPD. Yeast were diluted to $5x10^7$ cells/mL in fresh YPD and
2791 incubated in a vinyl anaerobic chamber (Type B; Coy Laboratory Products) maintained at
2792 37°C. The atmosphere of the chamber was filled with an anaerobic gas mix comprised of
2793 85% nitrogen, 10% hydrogen and 5% carbon dioxide, and was set up and operated as

2794 previously described [493]. One milliliter samples were taken over 24 hours to measure
2795 OD_{600} values. Samples were also taken at 12 and 24 hours for CFU counts.

2796 **5) Yeast Transformation**

2797 All yeast were transformed using standard electroporation [494] and LiOAc [431]
2798 protocols. Briefly, for LiOAc transformation, overnight cultures were diluted to $2x10^6$
2799 cells/mL in fresh YPD and incubated at 30°C until reaching a concentration of
2800 10^7cells/mL. Cells were then washed in sterile water and TE/LiOAc and combined with
2801 plasmid DNA, carrier DNA, and PEG/TE/LiOAc and agitated for 30 minutes at 30°C.
2802 DMSO was then added and cells were heat shocked at 42°C for 15 minutes, washed, and
2803 plated onto selective media. For electroporation, cells were grown overnight to saturation,
2804 diluted to an OD_{600} of 0.2 and incubated until reaching an OD_{600} of 1.6. Cells were
2805 washed with ice cold water and buffer containing 1 M sorbitol and 1 mM $CaCl_2$. Cells
2806 were resuspended in 100 mM LiOAc/10 mM DTT and agitated for 30 min at 30°C.
2807 Pelleted cells were then washed and resuspended in buffer containing 1 M sorbitol and 1
2808 mM $CaCl_2$. A 400 μL volume of cells was combined with DNA, then electroporated
2809 using a BioRad micropulser. Cells were then transferred to a 1:1 mixture of YPD and 1
2810 M sorbitol and incubated one hour at 30°C with agitation. Cells were plated onto media
2811 containing 1 M sorbitol.

2812 **6) Analysis of GFP Fluorescence**

2813 Images of untransformed yeast and yeast transformed with a *URA3* plasmid
2814 encoding GFP were collected using an Olympus IX80. Flow cytometry was performed by
2815 resuspending cells in FACS buffer (sterile PBS and 0.5% FBS) and analyzing them using
2816 a BD LSR II flow cytometer and B530/30 filter.

2817 **7) Isolation of Viable Yeast from Murine Peyer's Patches**

2818 All animal experiments were conducted strictly in adherence to the guidelines and
2819 recommendations in the National Institutes of Health Guide for the Care and Use of
2820 Laboratory Animals. Experiments were approved by the Emory University Institutional
2821 Animal Care and Use Committee (Protocol number: DAR-2002655-021817BN), and
2822 euthanasia was performed using CO_2. WT female C57BL/6J mice aged 6-8 weeks were
2823 gavaged 10^8 CFU of either WT *S. boulardii*, *S. cerevisiae* laboratory haploid, or *S.*
2824 *boulardii* Mutant 2. Peyer's patches from each mouse were harvested four hours post
2825 gavage, cell strained, and plated onto yeast media to detect viable colonies. YPD plates
2826 were used in plating of untransformed yeast and plates lacking uracil were used to select
2827 *S. cerevisiae* laboratory haploid and *S. boulardii* Mutant 2 transformed with a *URA3*
2828 plasmid. CFU were counted after 2-4 days incubation at 30°C. The sample size needed to
2829 determine a statistically significant difference was calculated using Lehr's formula
2830 $n=2(1.96+0.8416)^2/(d/s)^2$ where d is the smallest meaningful difference in means and s is
2831 the standard deviation of the observations in each group, assuming a power of 80% and a
2832 significance of 5% [495].

2833

2834 **c) Results**

2835 Numerous studies have characterized superior growth of *S. boulardii* relative to *S.*
2836 *cerevisiae* strains [309,310]. In order to test growth of *S. boulardii* (ATCC MYA-797™)
2837 compared to the *S. cerevisiae* strains used in this study (Table 7.1), yeast were incubated
2838 for 24 hours at either 30°C, the optimal growth temperature for most *S. cerevisiae* strains
2839 [333], or 37°C, normal human body temperature. Growth of *S. boulardii* was compared to

2840 three strains of *S. cerevisiae*. W303 was selected due its frequent use as an *S. cerevisiae*
2841 laboratory haploid strain. RM11-1a, an *S. cerevisiae* WT haploid strain that has been
2842 more recently isolated and which carries a lower rate of age related mutations relative to
2843 other *S. cerevisiae* strains, was used as a natural isolate comparison to *S. boulardii* [496].
2844 Finally, *S. cerevisiae* YH990, a diploid, was used to compare growth of *S. boulardii* to
2845 that of another diploid yeast strain.

2846 As shown in Fig 7.1, *S. boulardii* shows a faster rate of growth and higher
2847 saturation point at both 37°C and 30°C relative to all three *S. cerevisiae* strains tested
2848 (laboratory haploid, WT haploid, and diploid) in normal rich media (YPD). Although *S.*
2849 *boulardii* actually reaches a higher saturation point at 30°C versus 37°C, its superior
2850 growth at 37°C relative to *S. cerevisiae* indicates that *S. boulardii* is more likely to show
2851 better growth at body temperature than *S. cerevisiae*.

2852 **1) Diploid *S. boulardii* Require High Doses of UV Irradiation to Achieve**
2853 **50% Cell Survival**

2854 UV mutagenesis coupled with 5-FOA resistance was used to screen for *ura3*⁻ *S.*
2855 *boulardii* mutants. Previous UV mutagenesis studies have used high UV doses, resulting
2856 in only 10-20% survival, to screen for auxotrophic mutants [332,446]. Most of these studies
2857 targeted haploid *S. cerevisiae* strains; however, there has been only one report of tetrad
2858 formation and isolation of haploid *S. boulardii* cells [309]. Indeed, attempts in the present
2859 study to induce *S. boulardii* sporulation were unsuccessful. Higher doses of UV
2860 irradiation may thus be necessary to increase the likelihood of inducing homozygous
2861 mutations in both copies of *S. boulardii URA3*. However, in light of potential future *in*
2862 *vivo* applications, lower doses of irradiation would be optimal to avoid mutating genes

2863 related to *S. boulardii*'s superior growth and immunomodulatory characteristics. A 50%
2864 survival dose of UV irradiation was therefore chosen to screen for *ura3*⁻ *S. boulardii*
2865 mutants.

2866 To determine the UV dose necessary to kill 50% of *S. boulardii* cells, WT *S.*
2867 *boulardii* as well as *S. cerevisiae* strains laboratory haploid, diploid, *RAD1*, and UV
2868 sensitive *rad1* cells were exposed to UV irradiation (Fig 7.2). As expected, the *S.*
2869 *cerevisiae rad1* mutant was killed even with low doses (5,000 µJ) of UV irradiation.
2870 Comparing percent survival versus UV dose shows that, as expected, higher doses of UV
2871 irradiation are needed to kill WT *S. boulardii* and *S. cerevisiae* diploid cells relative to *S.*
2872 *cerevisiae* haploid *RAD1* cells. The UV dose corresponding to approximately 50% WT *S.*
2873 *boulardii* survival was determined to be 20,000-22,500 µJ and was used for subsequent
2874 screening for *S. boulardii ura3*⁻ mutants.

2875 **2) Isolation of Three *S. boulardii* Mutants Unable to Grow Without Uracil**

2876 Approximately 2.2×10^8 WT *S. boulardii* cells were irradiated at a 50% survival
2877 dose (20,000-22,500 µJ) and plated onto media containing 5-FOA (Fig 7.3A). Of these
2878 irradiated cells, approximately 2,200 were 5-FOA resistant. Eighty of these 5-FOA
2879 resistant colonies were further screened to confirm their ability to grow on plates
2880 containing 5-FOA and their inability to grow on plates or in liquid media lacking uracil
2881 (Fig 7.3B, C). As expected, WT *S. boulardii* can grow on YPD plates or plates lacking
2882 uracil but does not grow on plates containing 5-FOA. In contrast, *S. boulardii* Mutants 1,
2883 2, and 3 (M1, M2, and M3) show a pattern of growth similar to that of the laboratory
2884 haploid *ura3*⁻ *S. cerevisiae* strain, with the ability to grow on YPD plates or plates
2885 containing 5-FOA, but not on plates lacking uracil. Although the vast majority of

2886 colonies originally isolated from 5-FOA plates showed a high rate of reversion to a
2887 *URA3*$^{+}$ phenotype (approximately 1-2% reversion), *S. boulardii* Mutants 1-3 showed a
2888 relatively low rate of reversion (Fig 7.3D) comparable to that seen for a commonly used
2889 *ura3*$^{-}$ laboratory haploid *S. cerevisiae* strain. Indeed, *S. boulardii* M2 showed no
2890 detectable reversion.

2891 Sequence analysis of the *URA3* open reading frame in *S. boulardii* Mutants 1-3
2892 revealed single amino acid substitutions located outside of specific functional domains
2893 (Fig 7.4A). M1 and M3 both contained an A160S amino acid substitution, while M2
2894 contained an S81F amino acid substitution. Although simultaneous mutation of both
2895 copies of *URA3* in the diploid *S. boulardii* is likely to be an extremely rare event,
2896 selective pressure due to the presence of 5-FOA could have facilitated duplication of
2897 mutated *URA3*. Structural modeling of the *S. cerevisiae* Ura3 protein (PDB ID: 1DQX
2898 [497]) (Fig 7.4B, C) reveals that residue serine 81 is located within an α-helix. A change
2899 from serine (Fig 7.4C) to the larger phenylalanine (Fig 7.4D) at residue 81 could cause a
2900 steric clash with surrounding amino acids including phenylalanine 86 and leucine 87 on
2901 the opposing β strand, likely impairing proper protein folding and catalytic function. The
2902 reason for lack of Ura3 function in M1 and M3 is less clear than for M2. Residue 160 is
2903 located approximately 10 Å from the catalytic site and outside of any α-helices or β-
2904 pleated sheets (Fig 7.4B, E-F). Although residue 160 is conserved in *Homo sapiens*, *Mus*
2905 *musculus*, *Danio rerio*, and WT *S. boulardii* (Fig 7.4A) as alanine, the *S. cerevisiae* +D4
2906 Ura3 protein contains a serine at the homologous position [498]. Furthermore, the crystal
2907 structure of Ura3 from *S. cerevisiae* has been solved with an A160S substitution (Fig
2908 7.4B) (PDB ID: 1DQX [497]). These data suggests that mutations outside the open reading

2909 frame, such as in promoter or enhancer regions, might instead account for lack of Ura3
2910 function in M1 and M3.

2911 **3) *S. boulardii* Mutants are Resistant to Low pH and Bile Acid *In Vitro***

2912 As in previous studies[309,310], *S. boulardii* shows enhanced growth relative to *S.*
2913 *cerevisiae* strains in YPD as well as in media at pH 4, pH 8, and containing bile salts
2914 (0.3% OxGall) (Fig 7.5 A-D). In order to determine if *S. boulardii* Mutants 1-3 retained
2915 the characteristic ability of WT *S. boulardii* to withstand pH changes and bile acid, WT
2916 and mutant *S. boulardii* were grown in pH-adjusted media for 24 hours (Fig 7A, E-G).
2917 All three *S. boulardii* mutants grow similarly in media at pH 4, pH 8, and 0.3% OxGall
2918 and reach a similar optical density (OD_{600}) at saturation. Notably, growth of all three *S.*
2919 *boulardii* mutants in YPD is decreased relative to WT *S. boulardii* (OD_{600} of
2920 approximately 0.6 at saturation for *S. boulardii* mutants versus over 1.0 for WT *S.*
2921 *boulardii*) (compare Fig 7.5A to Fig 7.5E-G). However, growth of *S. boulardii* mutants in
2922 media containing 0.3% OxGall or media at pH 4 is decreased only slightly relative to
2923 mutant growth in YPD. Furthermore, *S. boulardii* mutants appear less affected by media
2924 at pH 8 relative to *S. cerevisiae* laboratory haploid and diploid cells. Although the *S.*
2925 *boulardii* mutant growth rate at pH 8 is decreased relative to growth in YPD, OD_{600} at
2926 saturation in media at pH 8 almost reaches that seen with mutant growth in YPD. In
2927 contrast, growth of *S. cerevisiae* laboratory haploid and diploid strains at pH 8 never
2928 reaches that seen in YPD. These results indicate that while the saturation point for growth
2929 of *S. boulardii* mutants is decreased relative to WT *S. boulardii*, pathways influencing pH
2930 and bile acid resistance have been maintained.

2931 **4) *S. boulardii* Mutants Show Increased Growth in Anaerobic Conditions**

2932 Given the ability of WT *S. boulardii* to grow in the anaerobic conditions of the
2933 gastrointestinal system, WT *S. boulardii* and Mutants 1-3 were incubated in anaerobic
2934 conditions for 24 hours (Fig 7.6). OD_{600} readings show that both WT and mutant *S.*
2935 *boulardii* grow more quickly and to a higher saturation point than the tested *S. cerevisiae*
2936 strains (lab haploid and diploid) (Fig 7.6A). Similarly, CFU counts of samples taken at 12
2937 and 24 hours incubation in anaerobic conditions show the highest number of viable cells
2938 for Mutants 1-3 followed by WT *S. boulardii*, with the lowest cell numbers for *S.*
2939 *cerevisiae* lab haploid and diploid (Fig 7.6B).

2940 **5) *S. boulardii* Mutants Can Be Transformed and Express Functional GFP**

2941 In order to determine whether the *S. boulardii* mutants can be successfully
2942 transformed and express heterologous protein, *S. boulardii* Mutants 1-3 were transformed
2943 with a *URA3* plasmid encoding GFP. Fluorescence microscopy reveals GFP fluorescence
2944 in transformed *S. boulardii* mutants and *S. cerevisiae* laboratory haploid cells, but no
2945 background fluorescence in untransformed yeast (Fig 7.7A). In addition, flow cytometry
2946 analysis shows a high percentage of GFP-expressing cells in transformed *S. cerevisiae*
2947 (44% v. 0.63% for transformed and untransformed yeast, respectively) and *S. boulardii*
2948 Mutant 2 (61.2% v. 0.68% for transformed and untransformed yeast, respectively) (Fig
2949 7.7B). These results demonstrate that *S. boulardii* mutants can express GFP as efficiently
2950 as the well characterized *S. cerevisiae* laboratory haploid strain.

2951 To test the ability of *S. boulardii* mutants to maintain plasmid and heterologous
2952 protein expression without selective pressure, as will occur in the gastrointestinal system,
2953 transformed yeast were incubated in YPD for 4 or 24 hours and subsequently tested for

2954 GFP expression. As shown by flow cytometry, transformed *S. cerevisiae* and *S. boulardii*
2955 Mutants 1-3 incubated in YPD for 4 or 24 hours maintained a high percentage of GFP-
2956 expressing cells comparable to that of yeast maintained in selective media lacking uracil
2957 (Fig 7.7C). GFP positive *S. boulardii* mutant cells also maintained comparable median
2958 fluorescence intensity after incubation in non selective YPD media, indicating that on
2959 average not only the number of cells but also GFP expression per cell was maintained
2960 over 24 hours without selective pressure.

2961 **6) Viable Transformed *S. boulardii* Mutant 2 Expressing GFP can be**
2962 **Isolated from Murine Peyer's Patches**

2963 Use of transformed mutant *S. boulardii* for delivery of recombinant protein to the
2964 intestine depends not only on the ability to maintain plasmid without selection but also on
2965 the ability to survive passage through the gastrointestinal tract. Furthermore, in the case
2966 of cytokine delivery, the ability to contact immune tissues of the small intestine will be
2967 critical in helping to induce anti-inflammatory responses. Peyer's patches are major sites
2968 of antigen sampling from the small intestine lumen as well as key sites of immune
2969 response induction and development [428]. Thus, uptake of transformed yeast into Peyer's
2970 patches would indicate the ability of yeast not only to survive passage through the
2971 gastrointestinal tract but also to contact tissues responsible for mediating immune
2972 responses. As all three *S. boulardii* mutants showed similar resistance *in vitro* to low pH
2973 and bile acid (Fig 7.5E-G) and to anaerobic conditions (Fig 7.6), *S. boulardii* Mutant 2
2974 was used for *in vivo* experiments in mice as this mutant has no detectable reversion to a
2975 *URA3*$^{+}$ phenotype (Fig 7.3D).

2976 To test for survival of *S. boulardii* Mutant 2 in the gastrointestinal tract, C57BL/6
2977 mice were gavaged with water (Naïve), 10^8 CFU untransformed WT *S. boulardii*, or *S.*
2978 *boulardii* Mutant 2 or 10^8 CFU *S. cerevisiae* laboratory haploid transformed with the
2979 *URA3* GFP plasmid (Fig 7.8A). Peyer's patches were harvested four hours post gavage,
2980 cell strained, and plated onto selective media lacking uracil. Peyer's patches from naïve
2981 mice were also plated onto YPD to check for the presence of contaminating yeast unable
2982 to grow in the absence of uracil. After 2-5 days incubation at 30°C, plates showed no
2983 viable yeast detected in Peyer's patches of naïve mice (Fig 7.8B, Naïve), few to no
2984 colonies of transformed *S. cerevisiae* (Fig 7.8B, *S.c.* GFP), and many viable colonies for
2985 both transformed *S. boulardii* Mutant 2 (M2 GFP) and untransformed WT *S. boulardii*
2986 (WT *S.b.*) (Fig 7.8B). These results are quantitated in Fig 7.8C. Viable transformed *S.*
2987 *boulardii* Mutant 2 furthermore showed a high percentage of GFP^+ cells, as determined
2988 by flow cytometry (Fig 7.8D). This result indicates that transformed *S. boulardii* Mutant
2989 2 is capable of maintaining heterologous protein expression despite the lack of selective
2990 pressure and harsh growth conditions within the gastrointestinal tract.

2991 Notably, there was a high degree of variability in number of viable CFU harvested
2992 per mouse (Fig 7.8C), especially for the WT *S. boulardii* and *S. boulardii* Mutant 2
2993 groups. As observed previously for recovery of viable WT *S. boulardii* versus *S.*
2994 *cerevisiae* Σ1278b and BY3 strains from murine Peyer's patches [309], this variability
2995 prevented the trend of increased viable *WT S. boulardii* and *S. boulardii* Mutant 2 versus
2996 *S. cerevisiae* from reaching statistical significance (power calculations indicate a sample
2997 size of greater than 200 mice is needed to determine a statistically significance between
2998 the Mutant 2 and *S. cerevisiae* groups, given an alpha of 0.05 and an expected significant

2999 difference of 5 CFU). Such variability could be due to numerous factors, including
3000 differences in feeding prior to oral gavage, differences in digestion and gastrointestinal
3001 motility, or margins of error in Peyer's patch dissection.
3002

3003
d) Discussion and Conclusions

3004 A major requirement for the development of *S. boulardii* as a viable drug delivery
3005 system is the development of strains that can maintain protein expression in the harsh
3006 digestive conditions of the gastrointestinal tract. Here, we employed UV mutagenesis and
3007 5-FOA screening to generate three *S. boulardii* auxotrophic mutant strains that can be
3008 genetically modified and transformed without reliance on antibiotic resistance markers.
3009 Critically, *S. boulardii* Mutants 1-3 can be transformed with *URA3* plasmids and express
3010 functional recombinant protein as demonstrated by GFP fluorescence (Fig 7.7A-B). *S.*
3011 *boulardii* Mutants 1-3 maintained expression of recombinant protein for 24 hours after
3012 removal of selective pressure (Fig 7.7C), a feature that will be especially important in the
3013 context of *in vivo* drug delivery. The ability of transformed mutants to continue
3014 producing GFP even after 24 hours without selective pressure suggests their potential to
3015 express and deliver recombinant proteins during transit through the gastrointestinal tract.
3016 *S. boulardii* has previously been used successfully to produce the mammalian
3017 anti-inflammatory cytokine interleukin 10 (IL-10) [20]. This study not only confirmed the
3018 ability of WT *S. boulardii* to successfully produce properly folded IL-10 as demonstrated
3019 by ELISA, but also showed *in vivo* functionality of this secreted cytokine. Oral gavage of
3020 transformed *S. boulardii* expressing IL-10 improved ulceration scores of mice in the
3021 dextran sodium sulfate (DSS) colitis model, although there was no difference in colonic

3022 thickening or histological score compared to controls. While this study provides proof of
3023 principle for expression of a therapeutically relevant recombinant protein by *S. boulardii*,
3024 transformation in this study was dependent on the presence of aminoglycoside resistance
3025 markers and growth in media supplemented with antibiotic. Our use of *ura3*$^-$ auxotrophic
3026 mutant strains of *S. boulardii* in the present study allowed for selection of transformants
3027 simply using media lacking uracil, obviating the need for antibiotic resistance markers.

3028 A goal is to genetically engineer *S. boulardii* strains to produce uracil auxotrophy
3029 without additional mutations that could impact desirable growth properties of this
3030 clinically used probiotic strain. Although a moderate dose of UV irradiation was selected
3031 in order to mutate *URA3* while limiting the number of additional mutations, genes other
3032 than *URA3* have likely been affected in *S. boulardii* Mutants 1-3. Indeed, overall growth
3033 of the three *ura3*$^-$ *S. boulardii* mutants is reduced relative to that of WT *S. boulardii* (Fig
3034 7.5). The exact mutations responsible for this reduced growth were not determined.
3035 Generation of a *ura3*$^-$ *S. boulardii* strain in future studies using a targeted approach would
3036 allow for creation of an auxotrophic mutant while maintaining pathways responsible for
3037 the superior growth rate and immunomodulatory properties of WT *S. boulardii*.

3038 Despite a modest impact on growth rate, the three *ura3*$^-$ *S. boulardii* mutants
3039 generated in this study show resistance *in vitro* to a wide range of pH, bile acid, and
3040 anaerobic conditions similar to that of the gastrointestinal tract (Fig 7.5, 7.6). Growth of
3041 Mutants 1-3 in anaerobic conditions is in fact higher than for WT S. boulardii and S.
3042 cerevisiae (Fig 7.6). Although growth of *S. boulardii* Mutants 1-3 in YPD is reduced
3043 relative to WT *S. boulardii* in aerobic conditions, their growth in low pH and bile acid
3044 relative to growth in normal YPD media is maintained. Resistance to low pH is a key

3045 feature of *S. boulardii*, distinguishing it from even closely related *S. cerevisiae* strains.
3046 Consistent with these findings, previous studies provide evidence that WT *S. boulardii*
3047 shows resistance to low pH [292]. Maintenance of resistance to low pH and anaerobic
3048 conditions could allow *S. boulardii* Mutants 1-3 not only to synthesize therapeutic
3049 proteins but also to serve as protective capsules for those proteins during transit through
3050 the gut. Use of these auxotrophic mutants for protein synthesis and packaging would
3051 decrease cost of oral drug development by eliminating the expensive steps of purifying
3052 and packaging proteins into capsules such as liposomes or nanoparticles.

3053 To assess the potential for *S. boulardii* auxotrophic mutants to serve as drug
3054 delivery vehicles, we tested their ability to maintain production of recombinant protein *in*
3055 *vivo*. For these oral gavage experiments, we selected *S. boulardii* Mutant 2, which
3056 showed equal or superior resistance to low pH, bile acid, and anaerobic conditions
3057 relative to the other mutants (Fig 7.5, 7.6), comparable production of GFP *in vitro* (Fig
3058 7.7A-C), and reversion to a *URA3*$^{+}$ phenotype at a rate below the limit of detection (Fig
3059 7.3D). Viable *S. boulardii* Mutant 2 was isolated within murine small intestine Peyer's
3060 patches at a frequency similar to that of WT *S. boulardii* (Fig 7.8C), indicating that *in*
3061 *vivo* survival through the gastrointestinal tract and uptake into small intestine immune
3062 tissues is maintained for this mutant and supporting the *in vitro* data showing that this
3063 mutant is resistant to low pH and bile acid (Fig 7.5). High levels of GFP expression in
3064 transformed *S. boulardii* Mutant 2 recovered from Peyer's patches furthermore indicate
3065 that this mutant is capable of delivering recombinant protein to the gastrointestinal tract
3066 and its associated immune tissues (Fig 7.8D). In fact, our results in this experiment likely
3067 underestimate the number of *ura3*$^{-}$ *S. boulardii* Mutant 2 cells entering the Peyer's

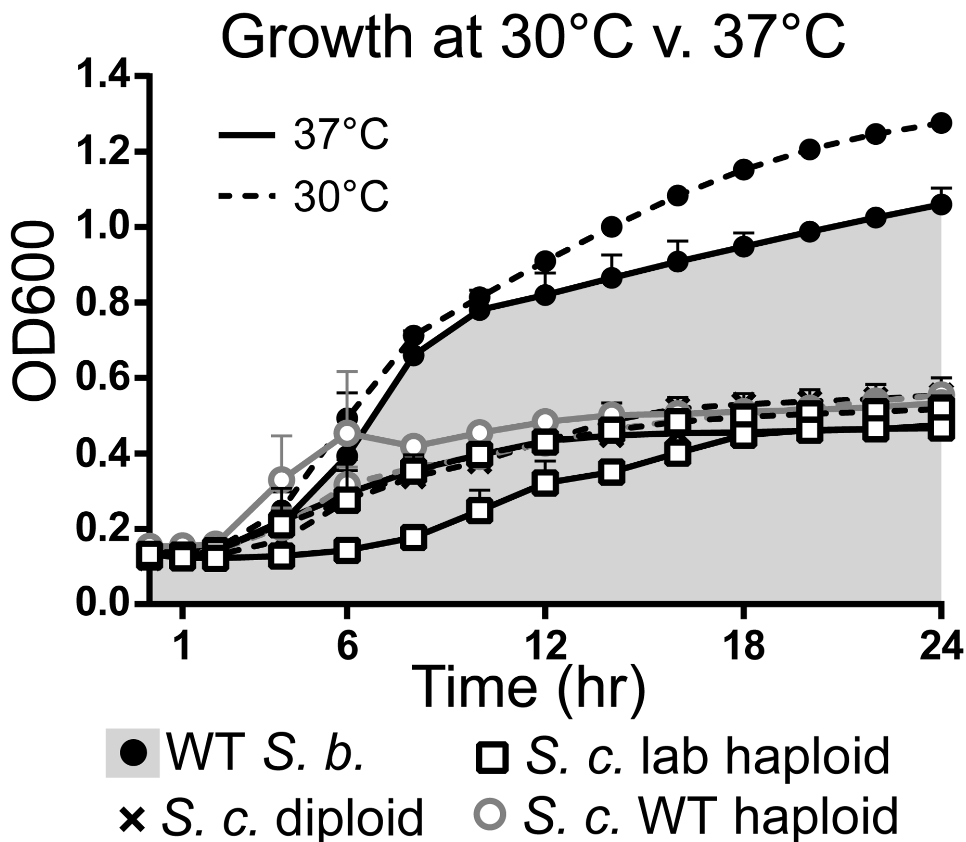
patches as our assay was capable of detecting only viable yeast; *ura3⁻ S. boulardii* that entered as cellular fragments or that were phagocytosed by antigen presenting cells within Peyer's patches could not be detected. The ability of *ura3⁻ S. boulardii* to be taken up into immune tissues makes it well suited for oral delivery of immunomodulatory therapeutics. For example, *ura3⁻ S. boulardii* could be used to express and deliver IL-10 to immune tissues of the gastrointestinal tract and thus promote anti inflammatory immune responses in the context of inflammatory bowel disease.

In summary, the *ura3⁻ S. boulardii* mutants generated in this study possess all the characteristics needed for safe and efficient use as an oral drug delivery system. These mutants can be transformed and selected using auxotrophic markers to avoid reliance on antibiotic selection. Furthermore, they can express heterologous protein to a similar level as a commonly used laboratory *S. cerevisiae* strain, as demonstrated by comparable levels of GFP fluorescence. These *S. boulardii* mutants also maintain high levels of protein expression even after prolonged incubation in nonselective media and uptake into the immune tissues of the murine gastrointestinal tract. These newly generated *S. boulardii* auxotrophic mutants are therefore good candidates for further testing as drug delivery vehicles for the treatment of gastrointestinal disorders.

e) Acknowledgements

We would like to thank Gray F. Crouse and Natalya P. Degtyareva for their generous contribution of yeast strains RM11-1a, SND 711, SND 713, and YH990 as well as for valuable discussions. We would also like to acknowledge Graeme L. Conn and William H. Hudson for helpful comments and discussions.

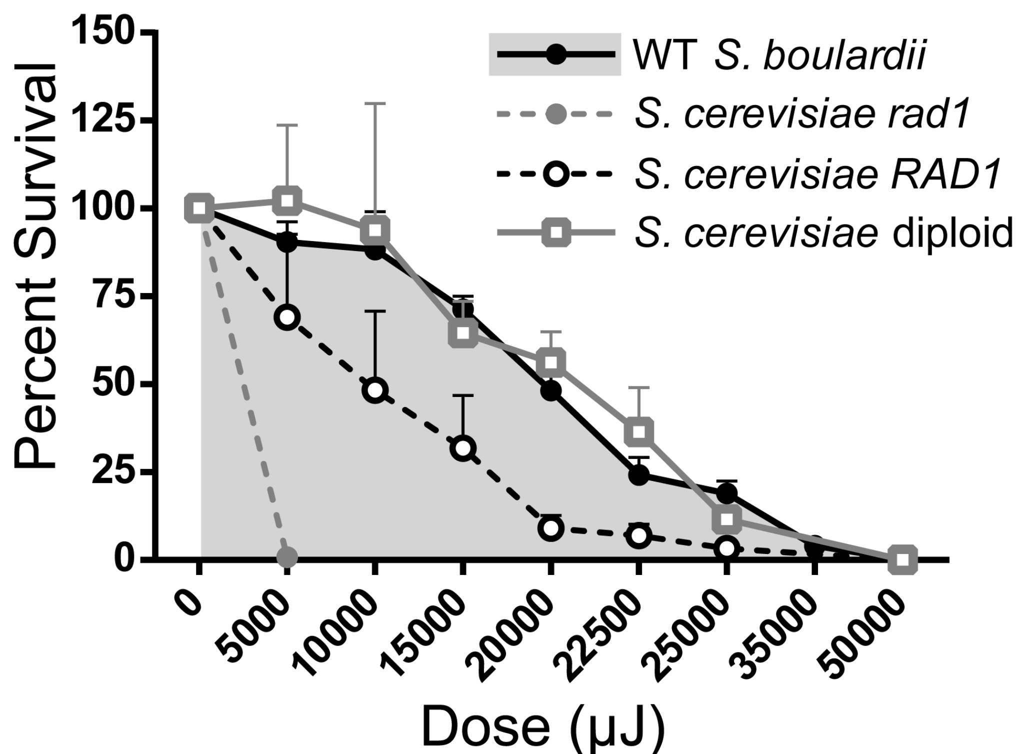
3092 **f) Figures and Tables**



3093
3094

3095 **Fig 7.1 *S. boulardii* Shows Enhanced Growth Relative to *S. cerevisiae* at Both 30°C**
3096 **and 37°C**

3097 Yeast were grown overnight in YPD and diluted to 10^7 cells per well in a 96 well
3098 plate. OD_{600} readings over 24 hours incubation at 37°C or 30°C indicate relative
3099 growth of wild type *S. boulardii* (WT *S.b.*), *S. cerevisiae* laboratory haploid (*S.c.* lab
3100 haploid), *S. cerevisiae* wild type haploid (*S.c.* WT haploid), and *S. cerevisiae* diploid
3101 (*S.c.* diploid). Lines represent the mean of duplicate experiments, with error bars
3102 depicting plus the standard error of the mean (SEM). Shading highlights growth of
3103 yeast strains relative to growth of W T *S. boulardii* at 37°C.



3104

3105 **Fig 7.2 Fifty Percent of *S. boulardii* Cells Survive at 20,000-22,500 µJ UV Irradiation**

3106 Wild type (WT) *S. boulardii* and *S. cerevisiae* diploid, haploid *RAD1*, and haploid *rad1*

3107 were exposed to various doses of UV irradiation. Percent survival (CFU as a percentage

3108 of total cells irradiated and plated) was plotted at each dose (mean of n=2 per strain per

3109 UV dose, with error bars depicting plus the standard error of the mean) to identify the

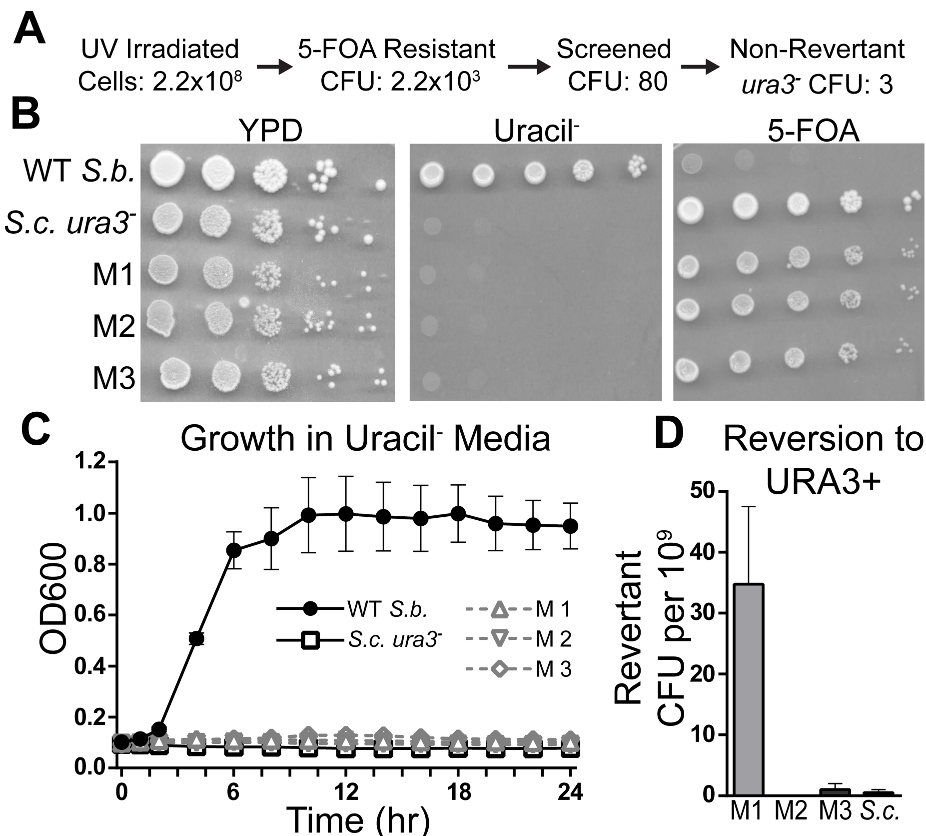
3110 dose of UV irradiation corresponding to 50% survival of WT *S. boulardii* cells. Greater

3111 than 100% survival was likely reached at some low UV doses due to cellular replication

3112 after irradiation.

3113

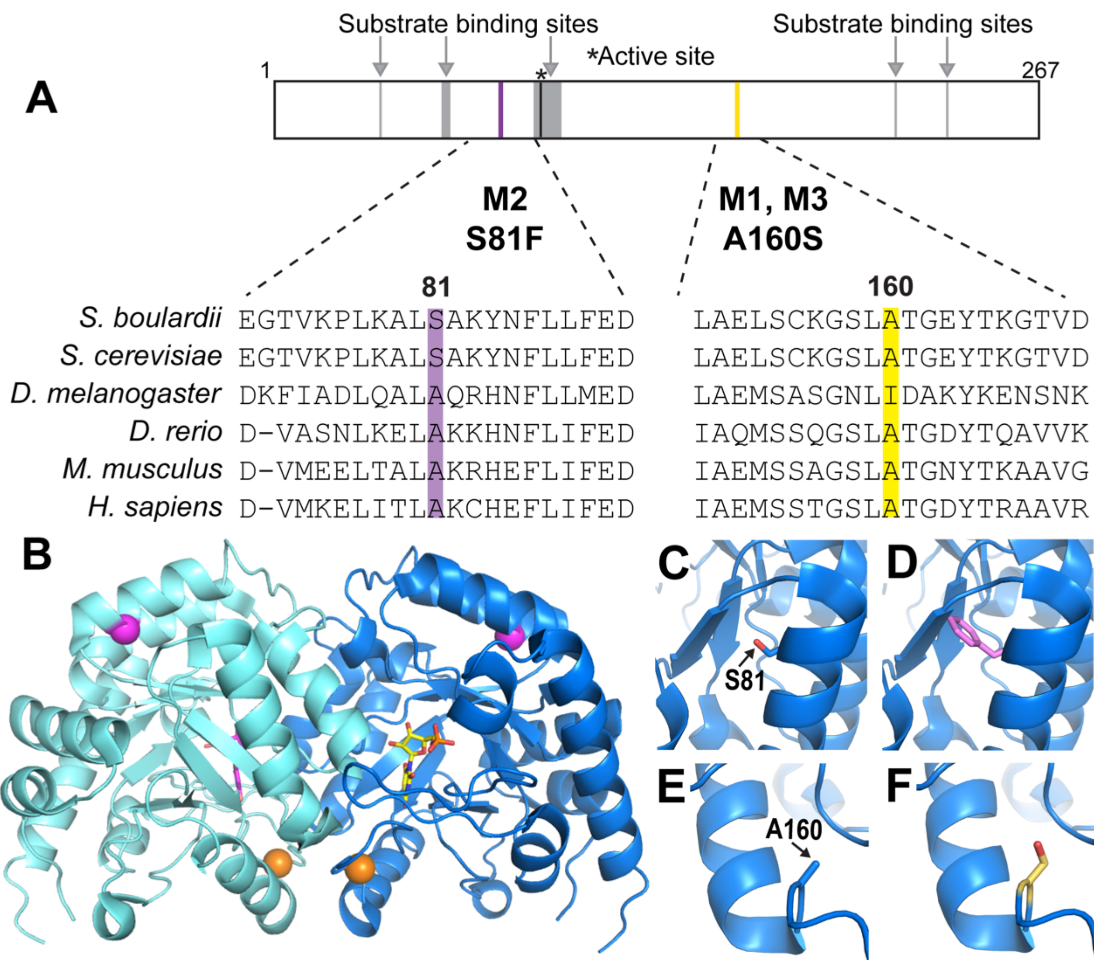
3114



3115

3116 **Fig 7.3 Isolation of Three *S. boulardii* Mutants Unable to Grow Without Uracil**

3117 (A) Flow diagram depicting the number of irradiated wild type (WT) *S. boulardii* cells,
3118 screened 5-FOA resistant colonies, and final number of *S. boulardii ura3⁻* mutants
3119 obtained. (B) Growth of WT *S. boulardii*, *S. boulardii* Mutants 1-3 (M1, M2, M3), and
3120 *ura3⁻ S. cerevisiae* laboratory haploid was assessed by serial dilution and spotting on
3121 YPD, uracil⁻, and 5-FOA plates. (C) Growth of *S. boulardii* Mutants 1-3 relative to WT *S.*
3122 *boulardii* and *ura3⁻ S. cerevisiae* laboratory haploid at 37°C in liquid media lacking
3123 uracil. Lines represent the mean of duplicate experiments for each strain, with error bars
3124 depicting plus and minus the standard error of the mean (SEM). (D) Number of CFU able
3125 to grow on plates lacking uracil per 10^9 plated cells. Each bar depicts the mean of
3126 duplicate experiments with error bars depicting plus the SEM.



3127

3128 **Fig 7.4 *S. boulardii ura3⁻* Mutants Contain Single Amino Acid Changes Within the**

3129 **Ura3 Protein**

3130 A) Schematic showing the domain structure of Ura3 protein in regions surrounding the

3131 amino acid changes in *S. boulardii ura3⁻* mutants. Ura3 substrate binding sites are shown

3132 in gray with arrows above (amino acids 37, 59-61, 91-100, 217, 235) and the active site

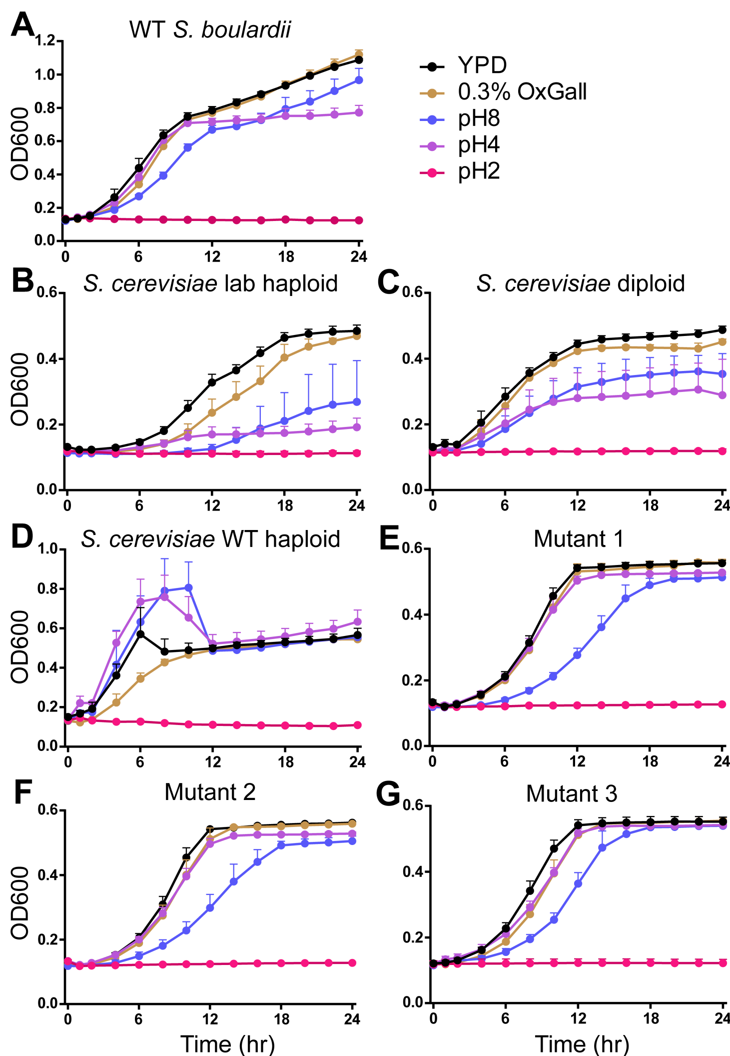
3133 as a black line with asterisk above (amino acid 93). The altered amino acid sites in the *S.*

3134 *boulardii* mutants are shown as purple (S81F in M2) and yellow (A160S in M1 and M3)

3135 lines with the changes indicated below. Homologous regions including the altered amino

3136 acids and the 20 surrounding residues in *Homo sapiens, Mus musculus, Danio rerio,*

3137 *Drosophila melanogaster, Saccharomyces cerevisiae*, and WT *S. boulardii* are depicted
3138 to show conservation of these residues. (B) Ribbon depiction of the *S. cerevisiae* Ura3
3139 homodimer bound to the proposed transition state analog 6-hydroxyuridine 5'-phosphate
3140 (PDB ID: 1DQX) [497]. The *S. boulardii* mutant single amino acid changes are noted in
3141 yellow (A160S in M1 and M3) and purple (S81F in M2). (C) Enlarged view showing the
3142 wild type serine residue at position 81. (D) Enlarged view showing the amino acid
3143 change to phenylalanine at position 81 in *S. boulardii* Mutant 2. (E) Enlarged view
3144 showing the wild type alanine residue at position 160. (F) Enlarged view showing the
3145 amino acid change to serine at position 160 in *S. boulardii* Mutants 1 and 3.
3146



3147

3148 **Fig 7.5 *S. boulardii ura3⁻* Mutants are Resistant to *In Vitro* Intestine-Like Conditions**

3149 Yeast were grown overnight in YPD and diluted to 10^7 cells per well in a 96 well plate.

3150 OD_{600} readings were taken over 24 hours incubation at 37°C. Graphs depict growth of

3151 yeast strains at pH 2, pH 4, pH 8, 0.3% OxGall, and YPD (approximately pH 6). Yeast

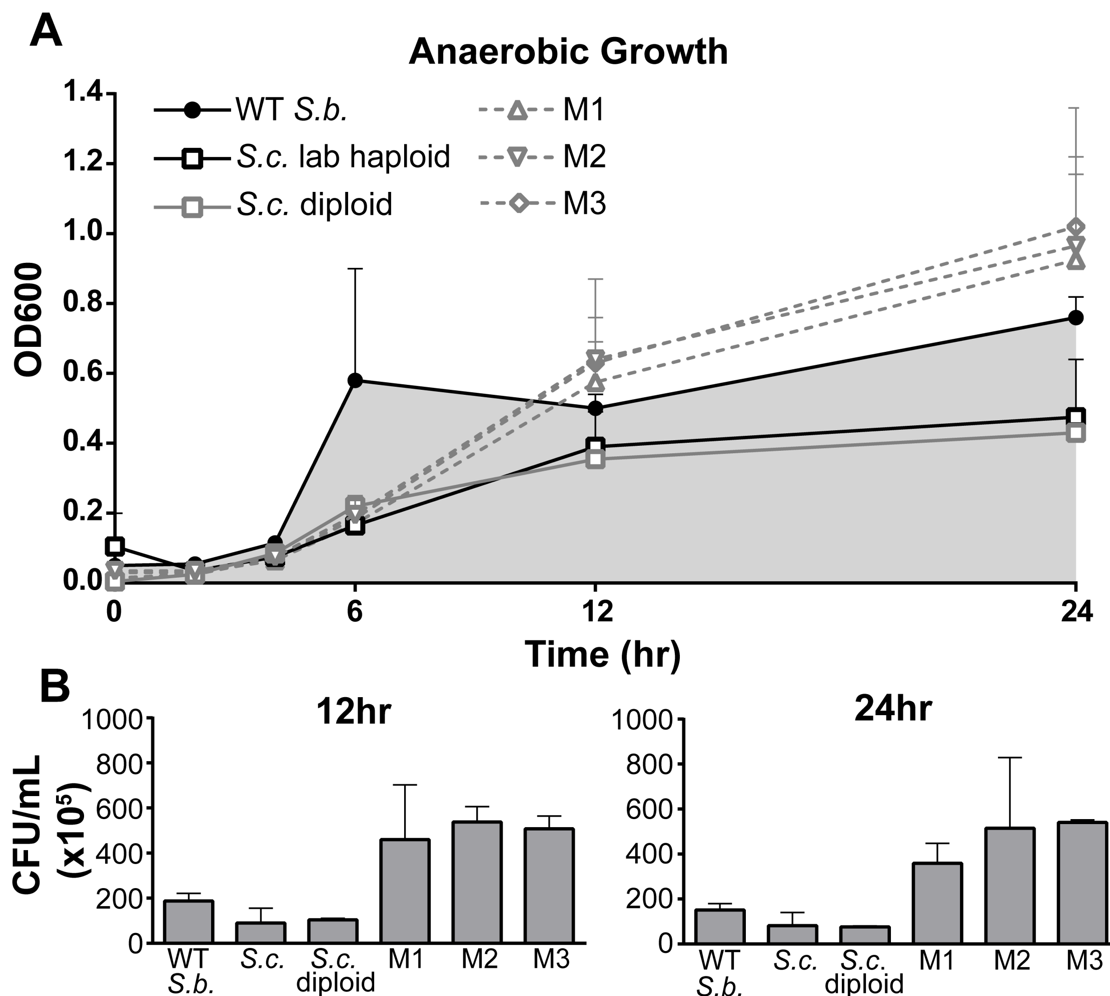
3152 strains include wild type (WT) *S. boulardii* (A); *S. cerevisiae* strains laboratory haploid

3153 (B), diploid (C), and wild type haploid (D); and *S. boulardii* M1 (E), M2 (F), and M3

3154 (G). This analysis shows that *S. boulardii* mutants maintain resistance to pH 4 and pH 8

3155 as well as to 0.3% OxGall whereas *S. cerevisiae* strains laboratory haploid and diploid are

3156 sensitive to these conditions.



3157

3158 **Fig 7.6 *S. boulardii ura3⁻* Mutants Grow in *In Vitro* Anaerobic Conditions**

3159 (A) Wild type (WT) *S. boulardii*; *S. cerevisiae* strains laboratory haploid, diploid, and

3160 wild type haploid; and *S. boulardii* M1, M2, and M3 were grown overnight in YPD

3161 and diluted to $5x10^7$ cells/mL in fresh YPD. OD_{600} readings were taken over 24

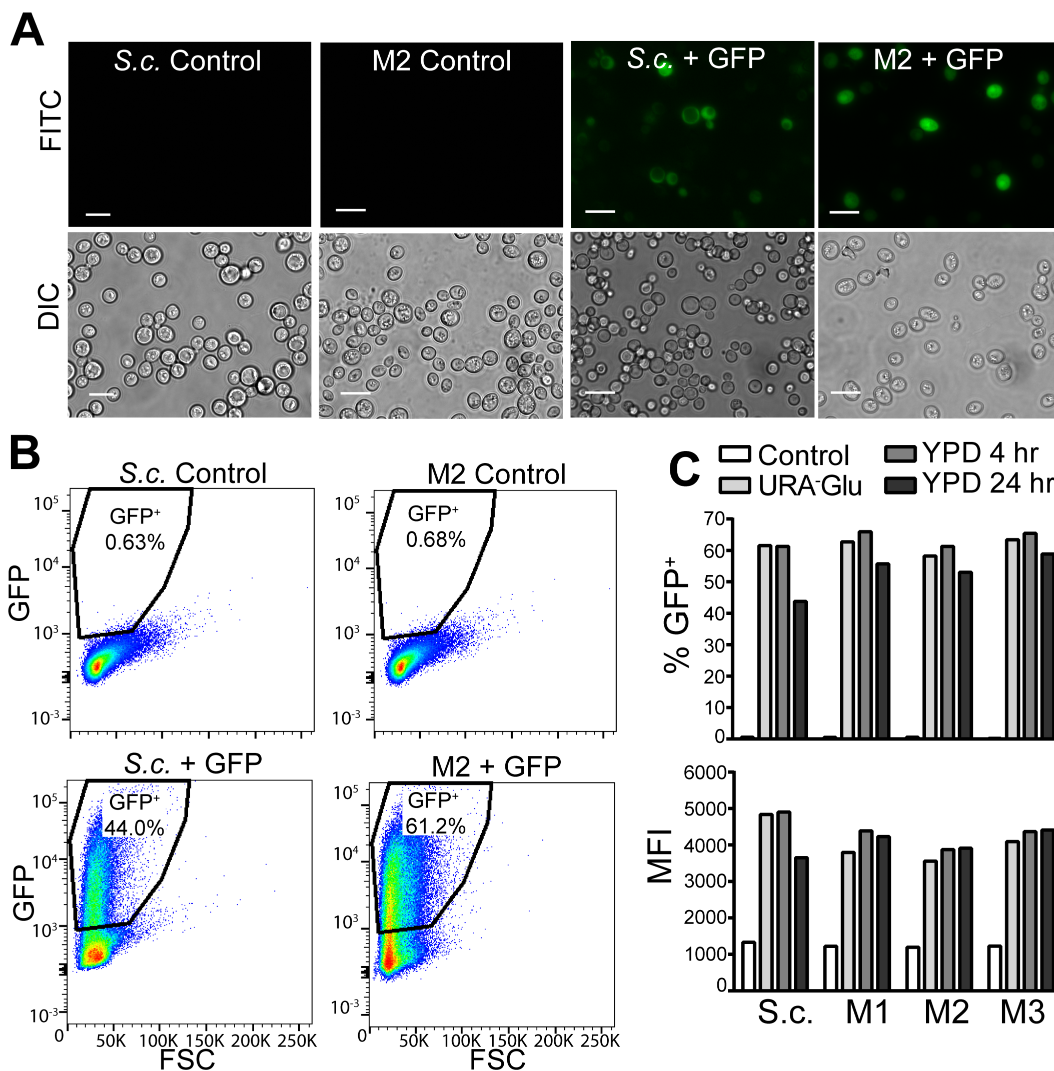
3162 hours incubation in a vinyl anaerobic chamber maintained at 37°C. (B) Number of

3163 colony forming units (CFU) per mL for each yeast strain after 12 and 24 hours

3164 incubation in the vinyl anaerobic chamber. This analysis shows that WT *S. boulardii*

3165 and particularly *S. boulardii* Mutants 1-3 show superior growth in anaerobic

3166 conditions relative to *S. cerevisiae* strains.

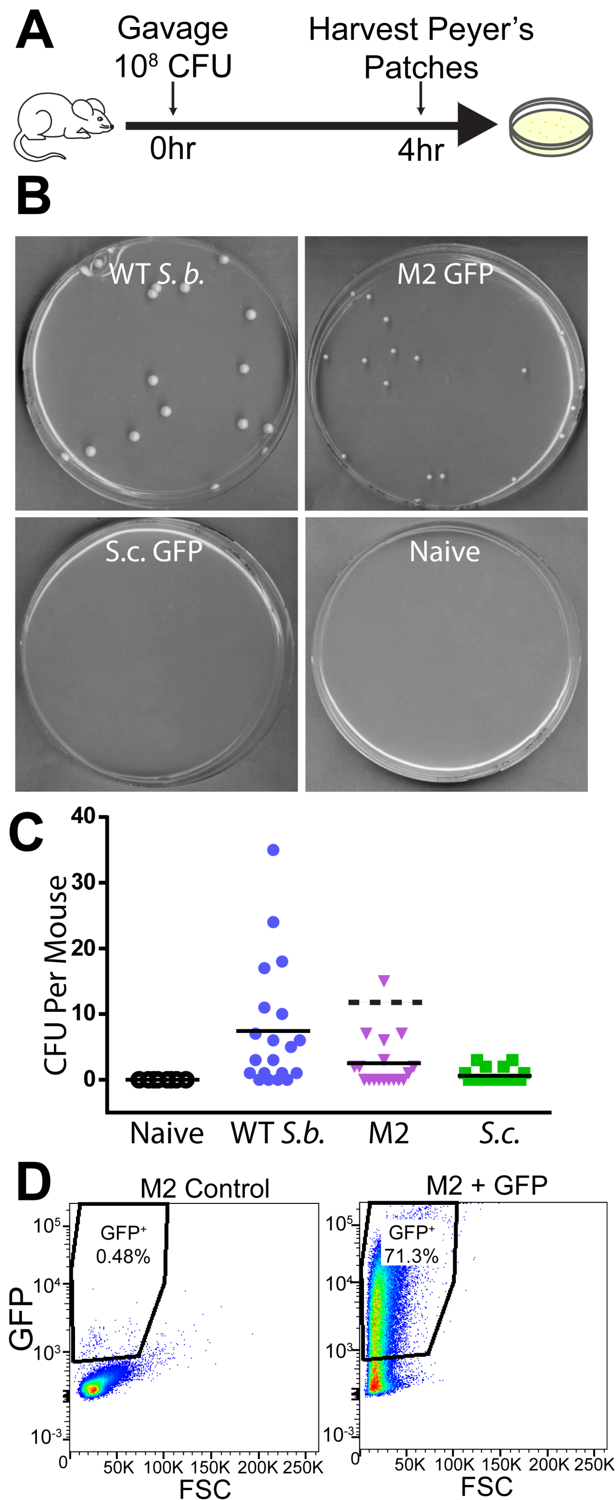


3167

3168 **Fig 7.7 *S. boulardii* Mutants Express Functional GFP**

3169 (A) Bright field and fluorescent images of *ura3⁻* *S. cerevisiae* laboratory haploid (*S.c.*)
3170 and *S. boulardii* Mutant 2 (M2) either untransformed (Control) or transformed (+ GFP)
3171 with a *URA3* plasmid containing GFP. The GFP fluorescence is detected in the FITC
3172 channel. Corresponding differential interference contrast (DIC) images are also shown.
3173 Scale bars show 10 µm. (B) Representative flow cytometry plots of forward-scattered
3174 light (FSC) versus GFP fluorescence for untransformed (Control) and transformed (+
3175 GFP) *S. cerevisiae* laboratory haploid (*S.c.* lab haploid) and *S. boulardii* Mutant 2 (M2)
3176 showing the percent of GFP positive cells in each population (n=2 per strain).

3177 Transformed yeast were maintained in media lacking uracil prior to analysis. (C)
3178 Retention of *URA3* plasmid and GFP expression was tested by comparing the percent of
3179 GFP positive cells of untransformed yeast (Control) relative to transformed yeast cultured
3180 in either selective media lacking uracil (URA^-Glu), YPD (non selective media) for 4
3181 hours (YPD 4 hr), or YPD for 24 hours (YPD 24 hr). Yeast strains analyzed include
3182 untransformed and transformed *ura3*$^-$ *S. cerevisiae* laboratory haploid (*S.c.*) and *S.*
3183 *boulardii* Mutants 1-3 (M1, M2, M3). Median fluorescent intensity (MFI) of GFP
3184 positive cells in each population is also depicted, indicating there is no visible decrease in
3185 average GFP expression per cell after incubation in YPD for 4 or 24 hours. Bars depict
3186 the mean of two samples per strain per incubation condition.
3187



3188

3189 **Fig 7.8 Viable Transformed *S. boulardii* Mutant 2 can be Recovered from**

3190 **Gastrointestinal Immune Tissue**

3191 (A) Schematic depicting oral gavage experiments. C57BL/6 mice were gavaged with 100
3192 µL containing either water, 10^8 CFU wild type *S. boulardii* (WT *S.b.*), 10^8 CFU *S.*
3193 *boulardii* Mutant 2 (M2), or 10^8 CFU *ura3$^-$* *S. cerevisiae* laboratory haploid (*S.c.*).
3194 Peyer's patches, sites of antigen sampling and immune response generation in the
3195 gastrointestinal tract (reviewed in [428]), were harvested 4 hours post gavage and plated to
3196 detect viable CFU. (B) Images of typical plates from oral gavage experiments showing
3197 recovery of viable yeast from Peyer's patches. Samples from mice gavaged with WT *S.*
3198 *boulardii*, *S. boulardii* Mutant 2 transformed with *URA3* plasmid, or *S. cerevisiae*
3199 laboratory haploid transformed with *URA3* plasmid were plated on media lacking uracil.
3200 Samples from naïve mice were also plated on YPD media to detect any contaminating
3201 yeast unable to grow without uracil. (C) CFU per mouse recovered from Peyer's patches
3202 of mice orally gavaged with water (Naïve), WT *S. boulardii* (WT *S.b.*), *S. boulardii*
3203 Mutant 2 (M2), or *S. cerevisiae* laboratory haploid (*S.c.)* (n=20 mice per group). Lines
3204 show the mean CFU per mouse for each group. Two data points for *S. boulardii* Mutant 2
3205 (87 and 110 CFU per mouse) are not depicted in order to allow better visualization of
3206 other data points. The mean without the two high points is 2.5 (shown in solid black line).
3207 The mean including the two points is 12.1 (shown in dotted line). (D) Representative
3208 flow cytometry plots of forward-scattered light (FSC) versus GFP fluorescence showing
3209 the percent of GFP positive cells among untransformed *S. boulardii* Mutant 2 (M2
3210 control) and *S. boulardii* Mutant 2 that was transformed with a *URA3* plasmid encoding
3211 GFP (M2 + GFP) and subsequently recovered from murine Peyer's patches (26 total
3212 transformed *S. boulardii* M2 CFU recovered from Peyer's patches were assessed by flow
3213 cytometry).

3214 **Table 7.1 Yeast Strains**

Strain	Designation	Description	Source
S. boulardii	WT *S. boulardii*	MYA-797	American Type Culture Collection
S. cerevisiae SND 713	*S. cerevisiae* rad1	rad1::kanMX; spore of hNDP223	[499]
S. cerevisiae SND 711	*S. cerevisiae* RAD1	WT spore of hNDP223	[499]
S. cerevisiae W303	*S. cerevisiae* laboratory haploid	MATα ura3Δ leu2Δ trp1Δ his3Δ	http://www.yeastgenome.org/
S. cerevisiae YH990	*S. cerevisiae* diploid	2n a/α by YEpHO-LEU2 of E134	[500]
S. cerevisiae RM11-1a (GCY 2860)	*S. cerevisiae* wild type haploid	MATα ho::loxP lys2Δ0 ura3Δ0	Generated by transient Cre expression to eliminate G418 resistance marker in strain UCC1159 [496]

3215

3216

8) Vaccine Delivery to the Murine Gastrointestinal Tract Using an Auxotrophic Mutant Strain of the Probiotic Yeast Sacc*haromyces boulardii*

a) Introduction

At the start of the experiments presented in this work, there was no available data exploring the ability of recombinant *S. boulardii* to induce antigen-specific mucosal immune responses against heterologously expressed peptides. Determining the appropriate gavage schedule and optimal adjuvant needed to stimulate responses in combination with *S. boulardii* will be an important step in further developing this probiotic yeast strain as a vaccine delivery vector. Here we show a series of pilot experiments, run concurrently with the experiments presented in Chapter 6, that test the ability of transformed auxotrophic mutant *S. boulardii* to induce antibody responses and protection against pathogen challenge in mice. As described in Chapter 7, use of the *S. boulardii* M2 mutant allows for efficient transformation without reliance on antibiotic selection while maintaining key characteristics of WT *S. boulardii* such as increased resistance to bile salts and pH stresses. Although these pilot experiments show limited host responses to recombinant *S. boulardii* vaccination, they provide useful information as to the feasibility of using auxotrophic *S. boulardii* to stimulate mucosal immunity and provide a basis upon which to further refine *S. boulardii* as a vaccine delivery vehicle.

These experiments involved tests of multiple antigens and adjuvants for induction of protective mucosal immune responses (summarized in Table 8.1). Both ovalbumin and a peptide fragment of the influenza A nucleocapsid protein (NP) were used as model

3240 antigens. The first adjuvant system tested, based on ImmunoBody™ technology
3241 (personal communications with Dr. Camilo Colaco, [501]), entailed antigen fusion to a
3242 portion of the mouse IgG2a heavy chain, including the hinge region and constant heavy
3243 chain regions 2 and 3 (CH2 and CH3). This system permits targeting of vaccine antigen
3244 to the Fc receptors of APCs, promoting phagocytosis and presentation of vaccine antigen
3245 [501] (Fig 8.1). Delivery of Fc in combination with CD4 and CD8 cancer epitopes or
3246 influenza hemagglutinin (HA) has also been shown to promote responses to
3247 subcutaneously delivered experimental cancer vaccines [502,503] or influenza HA [504] in
3248 mice. This system was thus selected due to its demonstrated effects as an adjuvant and its
3249 potential to be synthesized and expressed by yeast vaccines. An additional adjuvant was
3250 also used to evaluate the ability of transformed *S. boulardii* to induce protective immune
3251 responses against vaccine antigen. The *E. coli* heat labile toxin double mutant dmLT has
3252 been evaluated in numerous experimental oral vaccines [505–507] and has been shown to
3253 increase germinal center formation and antigen specific IgA secretion [506]. Oral
3254 administration of dmLT in combination with transformed yeast thus provided a means to
3255 test induction of antibodies specific for vaccine antigens expressed by *S. boulardii*.
3256

3257 ## b) Materials and Methods

3258 ### 1) Constructs and Cloning

3259 The vaccine constructs for chicken ovalbumin and the adjuvant Fc, which encodes
3260 a portion of the constant fragment of mouse IgG2a, were kind gifts from Dr. David
3261 Guiliano and Dr. Camilo Colaco. An antigen-adjuvant fusion construct was generated by
3262 inserting the Fc sequence immediately 3' to a myc-tagged chicken ovalbumin sequence

3263 (designated as Ova-Fc) and was cloned into the pRS426 *URA3* 2 µm yeast plasmid under
3264 the constitutive TEF1 promoter by the Emory University Cloning Core. Individual
3265 ovalbumin (Ova) and Fc constructs were also myc-tagged and cloned into pRS426 *URA3*
3266 plasmids under pTEF1 as controls. Vaccine constructs for the influenza study encoded
3267 amino acids 250-450 of the influenza A virus (A/Puerto Rico/8/1934(H1N1))
3268 nucleocapsid protein (NP). This sequence was also myc-tagged and cloned into pRS426
3269 *URA3 pTEF1* plasmid by the Emory University Cloning Core.

3270 **2) Yeast Strain and Transformation**

3271 All vaccine studies were conducted using the M2 *S. boulardii* $ura3^-$ auxotrophic
3272 mutant strain developed as previously described [408] (Chapter 7). *S. cerevisiae* W303 was
3273 also used for comparison in some transformation studies (http://www.yeastgenome.org/).
3274 Yeast were transformed using the LiOAc method as previously described [431] (Chapter 5).
3275 Briefly, overnight cultures incubated at 30°C were diluted to $2x10^6$ cells/mL in fresh
3276 YPD. Cells were returned to incubate at 30°C until reaching an approximate
3277 concentration of 10^7 cells/mL. Cells were then washed in sterile water and TE/LiOAc;
3278 combined with plasmid, carrier DNA, and PEG/TE/LiOAc; and incubated for 30 minutes
3279 at 30°C on a shaking incubator. DMSO was then added to the solution, and cells were
3280 heat shocked at 42°C for 15 minutes, washed, and plated onto selective uracil$^-$ media.

3281 **3) Immunoblotting**

3282 Transformed yeast were grown overnight to saturation in selective media and
3283 whole yeast were lysed by incubations at 100°C and -20°C. Precision Plus Protein
3284 WesternC or Kaleidoscope standards (BioRad) were used to determine approximate kDa
3285 weights. All constructs were myc tagged and detected using mouse anti myc (9B11) (Cell

3286 Signaling Technology) and goat anti mouse IgG IRDye 800CW (LiCor Biosciences)
3287 antibodies and a LiCor Odyssey InfraRed Imager.

3288 **4) Animal studies**

3289 Female C57BL/6 mice age 4-6 weeks obtained from Jackson Laboratories were
3290 maintained in sterile housing conditions. All studies were conducted with the approval of
3291 the Emory University Institutional Animal Care and Use Committee. Gavage schedules
3292 for vaccine studies (Fig 8.2) included alternating weeks of 100 μL daily doses of solution
3293 containing, as indicated, either vehicle control; purified chicken ovalbumin (Sigma); 10^8
3294 CFU untransformed yeast (M2); 10^8 CFU yeast transformed with plasmid to express myc
3295 tagged ovalbumin (M2 Ova), Fc (M2 Fc), the fusion Ova-Fc experimental vaccine
3296 constructs (M2 Ova-Fc), or the influenza nucleocapsid protein fragment (NP250-450); or
3297 10^8 CFU of NP250-450-expressing yeast admixed with 25 μg of the double mutant heat-
3298 labile *E. coli* toxin (dmLT) (R192G/L211A), a kind gift from Dr. John Clements.

3299 Experiments testing purified ovalbumin admixed with M2, as well as experiments
3300 testing heterologous expression of ovalbumin and the Fc adjuvant were performed
3301 according to the gavage regimen depicted in Fig 8.2A. Subsequent experiments using
3302 transformed M2 in conjunction with dmLT followed a slightly altered regimen depicted
3303 in Fig 8.2B. Blood samples were collected at indicated time points by cheek bleeds into
3304 heparinized tubes and spun at maximum speed in a microcentrifuge for 5 min at 4°C to
3305 collect serum. Fresh fecal pellets were prepared as previously described (Chapter 6).
3306 Mice were euthanized using isoflurane at the time points indicated. For influenza
3307 vaccination, mice were first immunized according the schedule in Fig 8.2C and then
3308 intranasally infected with a lethal strain of influenza A virus (A/Puerto

3309 Rico/8/1934(H1N1)). Mice were weighed and monitored for signs of distress daily, and
3310 were euthanized using isoflurane upon reaching 80% of baseline weight. Experiments are
3311 summarized in Table 8.1.

3312 **5) ELISA and ELISPOT**

3313 Assays for antibody and antibody secreting cells were performed as previously
3314 described (Chapter 6). Briefly, for ELISAs unlabeled goat anti mouse IgA and IgG
3315 (Southern Biotech) or 5 μg/mL filter sterilized chicken ovalbumin were used to coat
3316 plates overnight at 4°C in carbonate/bicarbonate buffer. Plates were blocked with TBST
3317 containing 5% nonfat dry milk for 2 hr at room temperature (RT), diluted samples were
3318 incubated overnight at 4°C, and secondary goat anti mouse IgA and IgG HRP-conjugated
3319 (Southern Biotech) antibodies were incubated for 1.5 hr at RT prior to addition of Super
3320 AquaBlue ELISA Substrate (eBiosciences) and reading at 405 nm. Purified mouse IgG
3321 (Invitrogen), IgA (BD biosciences), and anti-ovalbumin (Thermo Scientific) antibodies
3322 were used as standard controls.

3323 For ELISPOT, anti mouse IgG, IgA, IgM (Rockland) was diluted to 5 μg/mL in
3324 PBS to coat plates overnight at 4°C. Plates were blocked by 2 hr incubation at 37°C with
3325 complete IMDM before cell dilutions were incubated overnight at 37°C. Secondary
3326 biotin-conjugated anti mouse IgG and IgA antibodies (Southern Biotech) were diluted in
3327 PBST 1% FCS and incubated overnight at 4°C, then HRP avidin D (Vector Laboratories)
3328 in PBST 1% FCS was added to wells for 1-3 hr at room temperature. Color reactions
3329 using AEC substrate (0.3mg 3-amino-9-ethylcarbazole in 0.1 M Na-Acetate buffer, pH 5,
3330 0.3% hydrogen peroxide) were allowed to proceed for 2-10 minutes before washing with
3331 distilled water. Plates were counted with the aid of a CTL ImmunoSpot 5.1.36 analyzer.

3332 **c) Results**

3333 **1) Ovalbumin and Fc constructs can be expressed by *Saccharomyces***

3334 The *ura3*⁻ auxotrophic *S. boulardii* mutant M2 has already been shown to
3335 successfully express heterologous protein in the absence of antibiotic selection [408]. For
3336 proof of principle experiments testing the ability of this mutant *S. boulardii* to induce
3337 antibody responses to heterologously expressed antigen in mice, the well characterized
3338 antigen ovalbumin was selected. The nucleotide sequence encoding a 345 amino acid
3339 peptide of chicken ovalbumin, which excludes the secretion signal peptide, was myc
3340 tagged and cloned into a *URA3* 2 μm yeast plasmid under the expression of the strong
3341 constitutive yeast promoter TEF1 (plasmid henceforth abbreviated as Ova). To test the
3342 ability of M2 to simultaneously express a vaccine adjuvant, a sequence encoding a
3343 portion of the constant fraction of mouse IgG2a was myc tagged and cloned into the
3344 *URA3* pTEF1 yeast plasmid alone as a control (abbreviated as Fc) and 3' to the chicken
3345 ovalbumin sequence to create a fused antigen-adjuvant vaccine construct (Ova-Fc).
3346 *Saccharomyces cerevisiae* W303 transformed with these plasmids successfully expressed
3347 the myc tagged peptides at the expected kDa weights (Fig 8.3).

3348 **2) *S. boulardii* mutant admixed with purified ovalbumin does not induce**
3349 **significantly increased antibody responses**

3350 It is possible that *S. boulardii* itself may act as an adjuvant to stimulate antigen-
3351 specific responses without the need for expression of heterologous adjuvant. To
3352 determine the level of antibody induced by gavage with ovalbumin alone or by various
3353 concentrations of ovalbumin mixed with untransformed M2, mice were given a series of
3354 gavages of either vehicle control, 10^8 CFU untransformed M2, high dose purified chicken

3355 ovalbumin (2.5 mg Ova), or 10^8 CFU untransformed M2 with a range of purified
3356 ovalbumin concentrations (M2 + 2.5 mg, 250 μg, 25 μg, or 2.5 μg Ova). As shown in Fig
3357 8.2A, mice in each group received a total of 8 doses. However, ELISAs of fecal samples
3358 collected at various time points show no clear trend toward increased total IgA levels in
3359 any of the treatment groups (Fig 8.4). Furthermore, ELISPOT of whole tissues collected
3360 at week 8 shows high variability in the numbers of antibody secreting cells per 10^5 total
3361 cells in the Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleens of the
3362 different groups (Fig 8.5). Interestingly, numbers of IgA secreting cells in the MLNs
3363 appear to decrease with decreasing doses of ovalbumin combined with M2; however, this
3364 difference did not reach statistical significance. Higher numbers of IgG secreting
3365 splenocytes were detected in the M2 + 25 μg Ova group relative to other ovalbumin
3366 groups, but this too was not significantly different than the naïve group.

3367 **3) *S. boulardii* mutant expressing the ovalbumin vaccine construct does not**
3368 **induce significantly increased antibody responses**

3369 To test the ability of M2 to induce antibody response against heterologously
3370 expressed protein antigens, mice were gavaged with doses of 10^8 CFU M2 transformed
3371 with plasmid encoding the ovalbumin-Fc fusion construct (M2 Ova-Fc). Control groups
3372 were gavaged with either vehicle control (Naïve), 10^8 CFU untransformed M2 (M2), or
3373 10^8 CFU M2 expressing either the Fc fragment (M2 Fc) or ovalbumin (M2 Ova) only.
3374 Additional control groups were gavaged with purified ovalbumin to determine responses
3375 to antigen alone at a high 2.5 mg dose as well as at a lower 250 ng dose more reflective
3376 of levels potentially expressed by transformed M2. Groups were gavaged according to
3377 the schedule in Fig 8.2A, and M2 expression of experimental and control constructs was

3378 verified by Western blot (Fig 8.6). As shown in Fig 8.7, total antibody levels were not
3379 significantly increased with administration of M2 Ova-Fc relative to control groups.
3380 Although serum IgG showed a trend toward increased levels in the M2 Ova-Fc group
3381 relative to other control groups, this was not increased relative to the naïve group. This is
3382 also consistent with patterns seen for numbers of antibody secreting cells in the MLNs
3383 and spleens collected at week 8, as determined by ELISPOT (Fig 8.8). No clear
3384 differences were seen in the numbers of IgA or IgG secreting cells, although there were
3385 high levels of variability within groups. Finally, ELISA of serum collected at the week 8
3386 endpoint shows no difference in levels of ovalbumin-reactive IgA antibody; all groups
3387 had OD405nm values at background levels (Fig 8.9). Thus, no significant antibody
3388 responses were induced in mice receiving M2 expressing Ova-Fc relative to controls.

3389 **4) Addition of the mucosal adjuvant dmLT has a minimal effect on antibody**
3390 **responses in combination with transformed *S. boulardii* mutant**

3391 It is possible that lack of significant responses to test vaccine antigen expressed
3392 by M2 was due to improper expression or low efficacy of the proposed adjuvant Fc. To
3393 test an alternate, well characterized mucosal adjuvant in conjunction with recombinant
3394 M2, the *E. coli* double mutant heat labile toxin dmLT was added. Mice were gavaged
3395 with doses of either vehicle control (Naïve), 10^8 CFU M2 expressing ovalbumin (M2
3396 Ova) only, or 25 μg dmLT admixed with 10^8 CFU M2 expressing ovalbumin (M2 Ova +
3397 dmLT), interspersed by rest periods as demonstrated in previous studies using dmLT [506]
3398 (Fig 8.2B). Baseline levels of total IgA and IgG were similar across all groups (Fig 8.10),
3399 and although total antibody levels rose over time there were no significant differences
3400 between experimental groups. Assays to detect ovalbumin-reactive antibody by ELISA

3401 showed high overall background levels and no significant detectable differences in
3402 OD405nm values between groups, indicating that dmLT did not increase anti-ovalbumin
3403 antibody responses (Fig 8.10).

3404 To test the possibility that antibody responses in gavaged mice were generated
3405 against ovalbumin in an altered conformation, an additional ELISA was performed using
3406 plates coated with ovalbumin purified from transformed *S. boulardii* M2. Serum samples
3407 collected at the final time point were tested for the presence of ovalbumin-reactive IgA;
3408 however, as for ELISAs using plates coated with purified chicken ovalbumin, no
3409 OD405nm values were detected above background in any group (data not shown).

3410 **5) Vaccination with M2 expressing a nucleocapsid protein (NP) fragment**
3411 **fails to protect mice from lethal influenza challenge**

3412 Finally, to test the ability of M2 to induce responses against an alternate vaccine
3413 antigen, constructs encoding an influenza A virus (A/Puerto Rico/8/1934(H1N1)
3414 nucleocapsid protein (NP) fragment were cloned into the pRS426 *URA3 pTEF1* plasmid
3415 used for previous experiments. Previous studies have demonstrated extensive cross talk
3416 between the gastrointestinal and respiratory mucosa [508] as well as the ability of orally
3417 administered probiotic bacterial strains to boost protective responses to influenza
3418 vaccination [509–511] and to induce protective responses against heterologously expressed
3419 respiratory virus antigens [512]. Intranasal vaccination with NP in animal models is known
3420 to induce protective T cell responses against influenza challenge [513] and subsequent
3421 respiratory bacterial infection even in the absence of neutralizing immunity [514]. A few
3422 models have also tested delivery of NP via oral vaccination, including with live
3423 attenuated *Salmonella* [515] and immune stimulating complexes (ISCOMs) [516].

3424 In the present experiment, the sequence encoding NP amino acids 250-450 was
3425 used as this includes known CD4 and CD8 T cell epitopes in amino acids 311-325 and
3426 366-374, respectively. Transformed M2 was found to successfully express myc-tagged
3427 NP250-450 by immunoblot analysis (Fig 8.11). Mice were vaccinated in three doses,
3428 each with three consecutive daily gavages of either vehicle control (Naïve), 10^8 CFU M2,
3429 10^8 CFU M2 expressing NP250-450 (M2 NP250-450), or 25 µg dmLT admixed with 10^8
3430 CFU M2 expressing NP250-450 (M2 NP250-450 + dmLT) (Fig 8.2C). Upon intranasal
3431 infection with a lethal strain of Influenza A H1N1, mice were monitored daily for weight
3432 loss and signs of distress (Fig 8.12). Mice lost weight precipitously starting at
3433 approximately day 2. By day 5 all groups of mice had reached 80% of baseline weight
3434 with no significant differences among vaccinated versus control groups. Thus, mice
3435 vaccinated with a combination of dmLT and M2 expressing amino acids 250-450 of
3436 influenza NP were not protected from challenge with this highly pathogenic influenza
3437 strain.

3438

3439 **d) Discussion**

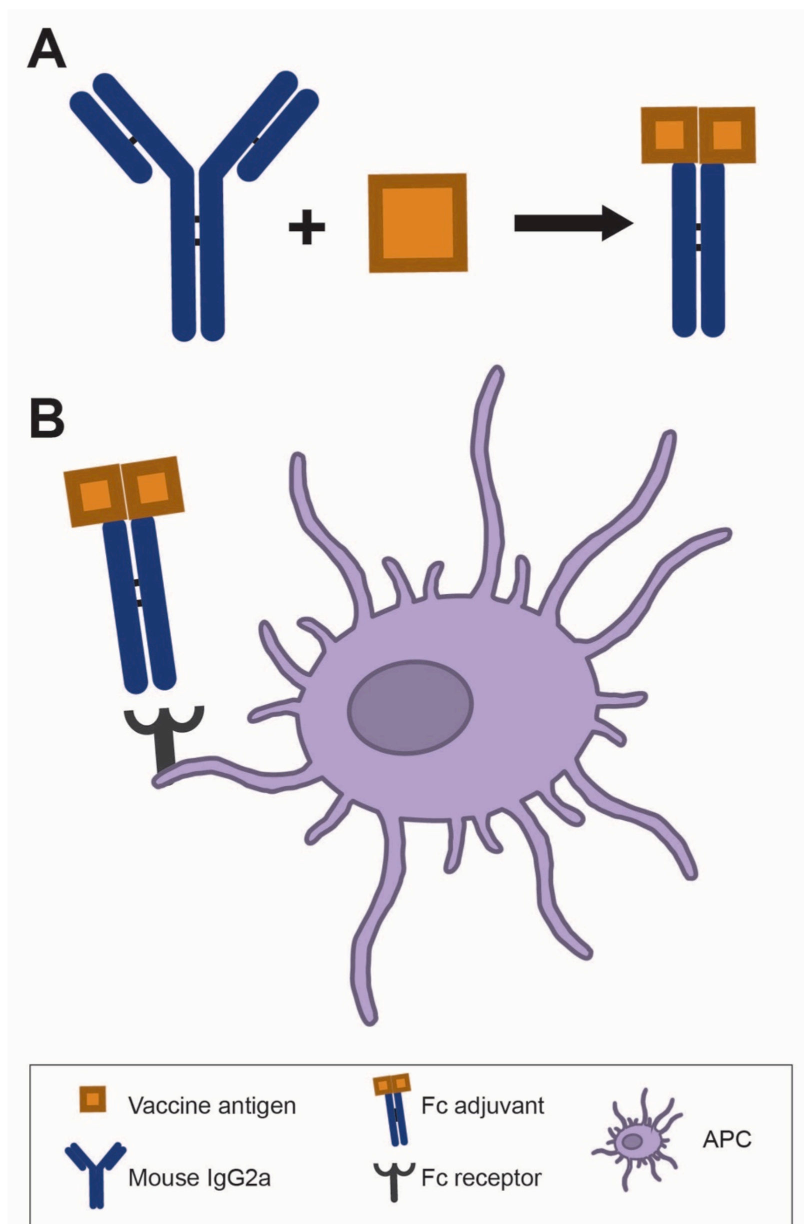
3440 The vaccination studies presented in this chapter demonstrate that although the *S.*
3441 *boulardii* auxotrophic M2 mutant is able to express heterologous model vaccine antigens,
3442 it is not able to induce antigen specific antibody responses. Experiments testing induction
3443 of mucosal immune responses to ovalbumin expressed by M2, in combination with the
3444 novel adjuvant Fc or the mucosal adjuvant dmLT, showed no difference relative to
3445 control mice gavaged with untransformed M2 or vehicle. Given the success of dmLT to
3446 induce protective mucosal immune responses in numerous other experimental systems, it

3447 is possible that alternate antigens would improve induction of antigen specific responses.
3448 Although oral vaccination with M2 expressing an influenza nucleoprotein peptide
3449 (NP250-450) in combination with dmLT failed to protect mice from challenge with a
3450 lethal influenza strain, it is possible that specific responses to NP, if present, were unable
3451 to prevent the severe and rapid decline seen after this particular challenge. Indeed, other
3452 studies suggest protection against influenza is improved with vaccination against both NP
3453 and hemagglutinin (HA). Use of additional antigens such as HA in an M2-based
3454 vaccination system, followed by testing for antigen specific B and T cell responses, may
3455 thus better indicate the potential of this system to mediate protection against pathogen
3456 challenge.
3457

3458 **e) Acknowledgements**

3459 Many thanks to members of both the Lamb and Corbett laboratories who assisted
3460 with these pilot vaccination experiments: Courtney McDermott, Taryn Stewart, Dr. Milo
3461 Fasken, Thayer King, Nate Jacobs, Dr. Patrice Mimche, Dr. Sarah Leung, Jessie Barra,
3462 Christian Bray, and Shelby Fruge, as well as Dr. Anita Corbett and Dr. Tracey Lamb. We
3463 also thank Dr. David Guiliano and Dr. Camilo Colaco for helpful discussions and
3464 provision of sequences for test vaccine constructs. We thank Dr. Jacob Kohlmeier and
3465 Dr. John Steel for helpful discussions and sequences for influenza vaccination
3466 experiments. Finally, we thank Dr. John Clements for kind provision of the dmLT
3467 mucosal adjuvant.
3468

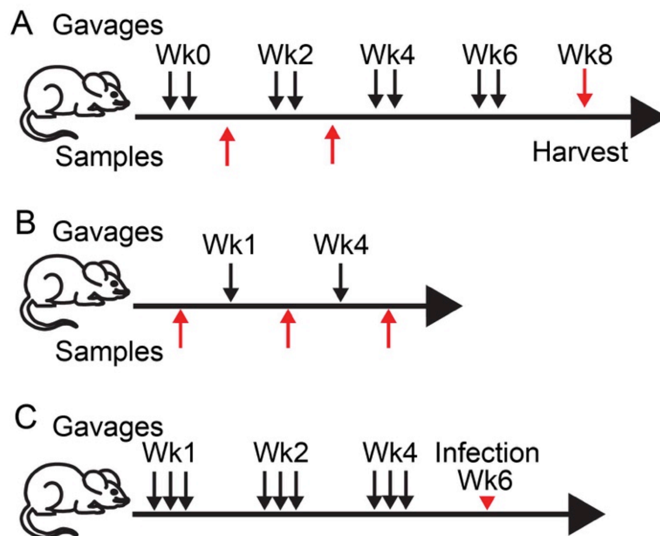
3469 **f) Figures and Tables**



3470
3471 **Fig 8.1 The Novel Fc Adjuvant Encodes the Fc Portion of Mouse IgG2a**

3472 (A) The novel Fc adjuvant expressed by *S. boulardii* M2 entails fusion of the sequences
3473 encoding the vaccine antigen (Ova) and the constant fragment of mouse IgG2a. (B) The
3474 Fc adjuvant is designed to ligate Fc receptors on antigen presenting cells (APCs) in order
3475 to facilitate antigen uptake and presentation.

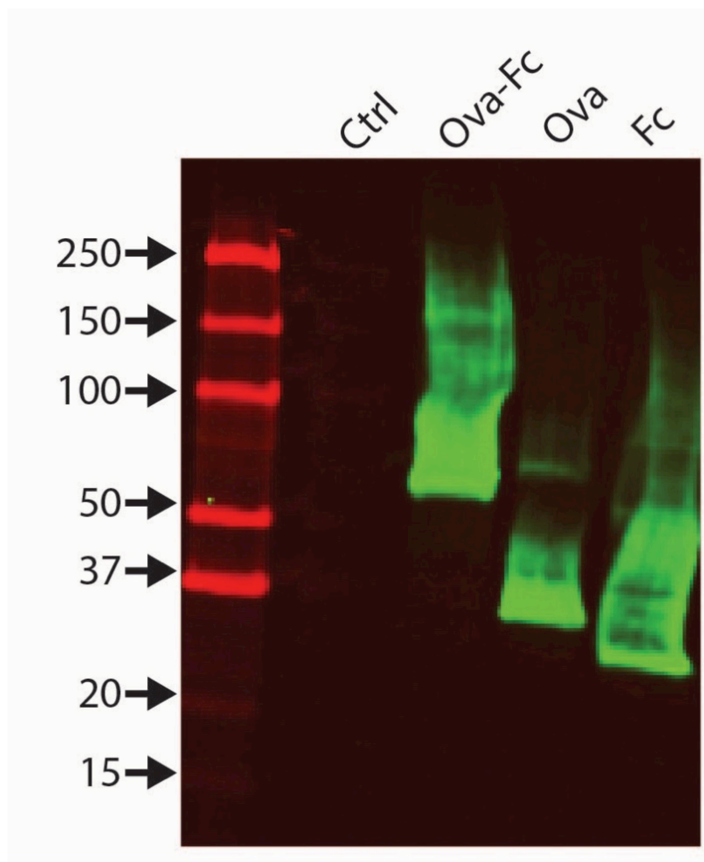
3476



3477

3478 **Fig 8.2 Gavage schedules for vaccine experiments**

3479 For gavage experiments testing purified ovalbumin admixed with *S. boulardii* M2 and
3480 experiments testing transformed M2 expressing ovalbumin and Fc adjuvant, the gavage
3481 schedule shown in (A) was used. For experiments testing the dmLT adjuvant in
3482 combination with M2 transformed to express ovalbumin, mice were gavaged according to
3483 the schedule shown in (B). For vaccination prior to challenge with influenza infection,
3484 mice were gavaged as in (C). In each experiment, C57BL/6 mice were gavaged (black
3485 arrows) with 100 µL of various solutions as indicated in the text, including: vehicle; 10^8
3486 CFU untransformed M2; purified ovalbumin; 10^8 CFU M2 and purified ovalbumin; 10^8
3487 CFU M2 transformed to express Ova, Fc, Ova-Fc, or NP250-450; or dmLT mixed with
3488 10^8 CFU M2 transformed to express NP250-450. Blood and fecal samples were collected
3489 (red arrows) at time points before and after gavage doses as shown (A and B). End point
3490 tissue samples were collected at week 8 (A). Mice were intranasally infected with
3491 influenza A virus (A/Puerto Rico/8/1934(H1N1)) on week 6 (red arrowhead in C).
3492 Experiment antigens and adjuvants are also summarized in Table 8.1.



3493

3494 **Fig 8.3. *Saccharomyces* successfully expresses Ova and Fc constructs**

3495 *S. cerevisiae* W303 was transformed with empty *URA3* plasmid or plasmid encoding the
3496 myc-tagged Ova, Fc, or Ova-Fc constructs under the control of the TEF1 constitutive
3497 yeast promoter. Immunoblot of crude whole yeast lysates from saturated cultures shows
3498 successful expression of the Ova, Fc, and Ova-Fc proteins of approximately 40, 30 and
3499 70 kDa, respectively. No myc tagged proteins were detected from yeast transformed with
3500 empty vector (Ctrl).

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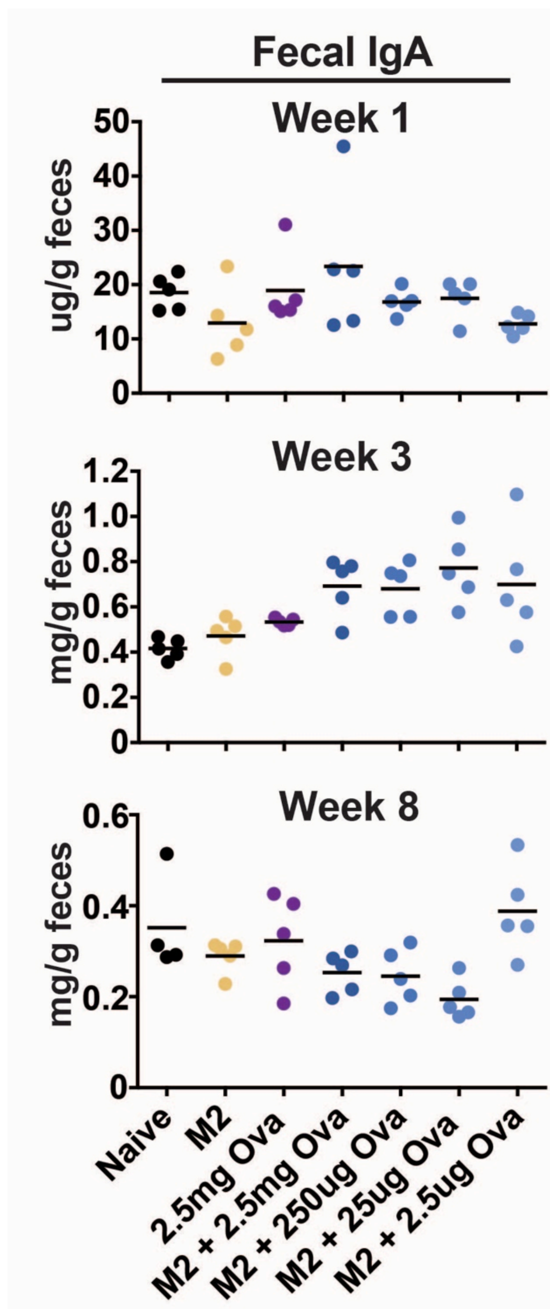

3505

3506 **Fig 8.4 Combined ovalbumin and M2 have little effect on secretory IgA levels**

3507 Total IgA levels were measured by ELISA in fecal samples collected on weeks 1, 3, and

3508 8 after 2, 4, and 8 total gavages, respectively. Plots depict n = 5 mice per group in one

3509 independent experiment.

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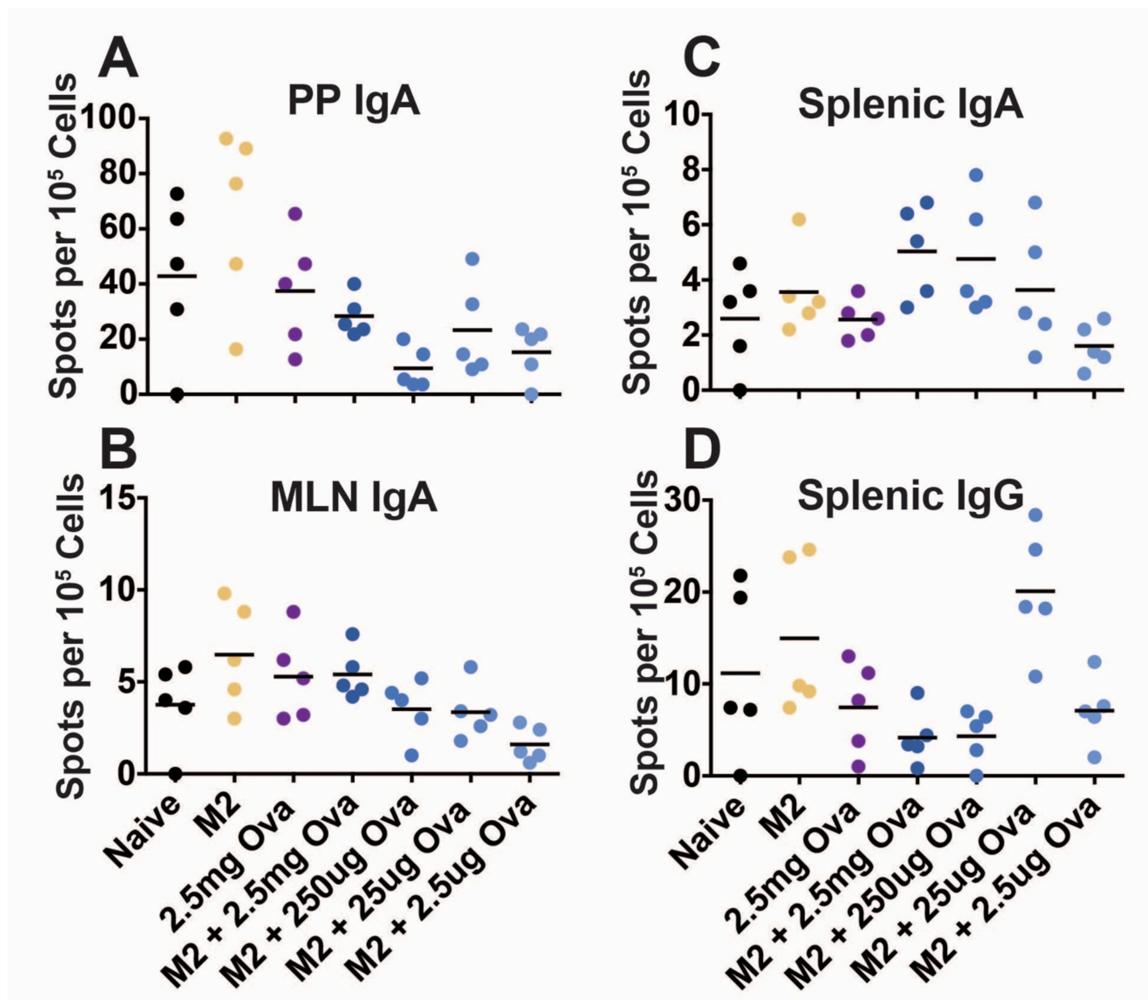
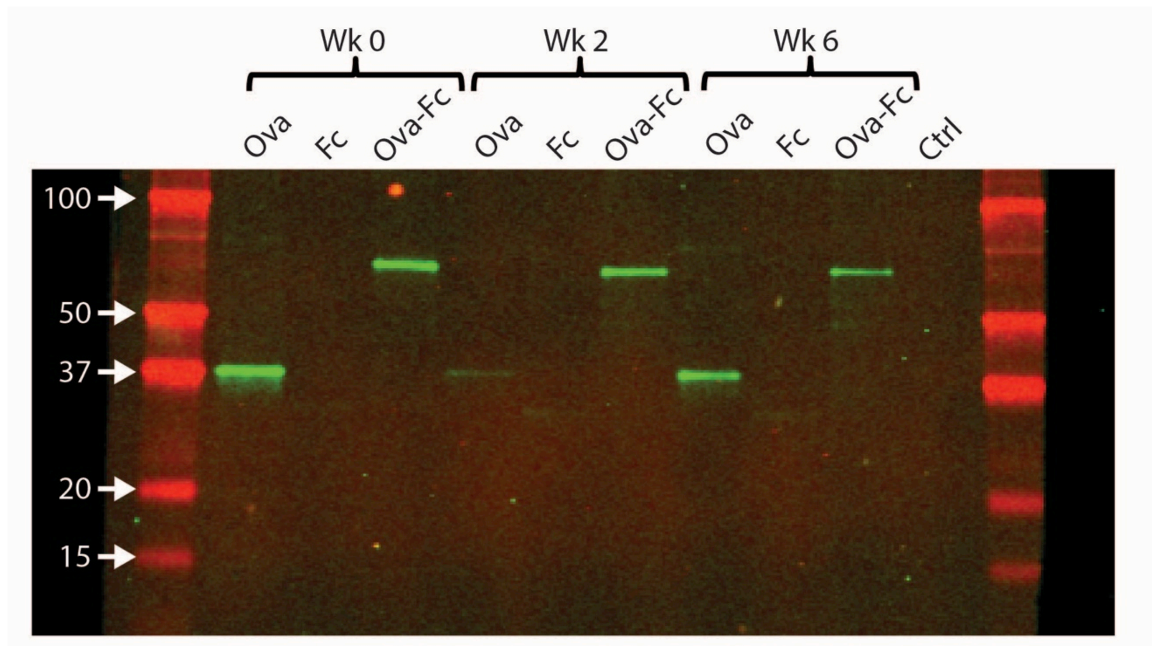


3511
3512 **Fig. 8.5 Combined ovalbumin and M2 have little effect on numbers of antibody**
3513 **secreting cells**

3514 ELISPOT shows only subtle differences in the numbers of IgA and IgG secreting cells in
3515 Peyer's patches (PP) (A), mesenteric lymph nodes (MLN) (B), and spleens (C, D)
3516 collected at harvest (n = 5 mice in each group in one independent experiment).



3517

3518 **Fig 8.6 Transformed M2 cultures express vaccine constructs**

3519 Aliquots of transformed M2 cultures used in gavages were collected on weeks 0, 2, and 6
3520 and stored in glycerol at -80°C. Yeast were later washed and lysed, and 30 ug of purified
3521 total protein per sample were run on a protein gel. Protein from an untransformed M2
3522 culture was also run as a negative control (Ctrl). Primary anti myc and secondary anti
3523 mouse IRDye-conjugated antibodies and an Odyssey LiCor Infrared imager were used
3524 for detection, and the Kaleidoscope protein standard was used to estimate kDa weight.
3525 Bands corresponding to approximately 40, 30, and 70 kDa were detected in samples of
3526 M2 transformed to express Ova, Fc, and Ova-Fc, respectively. Thus, mice indeed
3527 received M2 expressing the appropriate experimental vaccine constructs.

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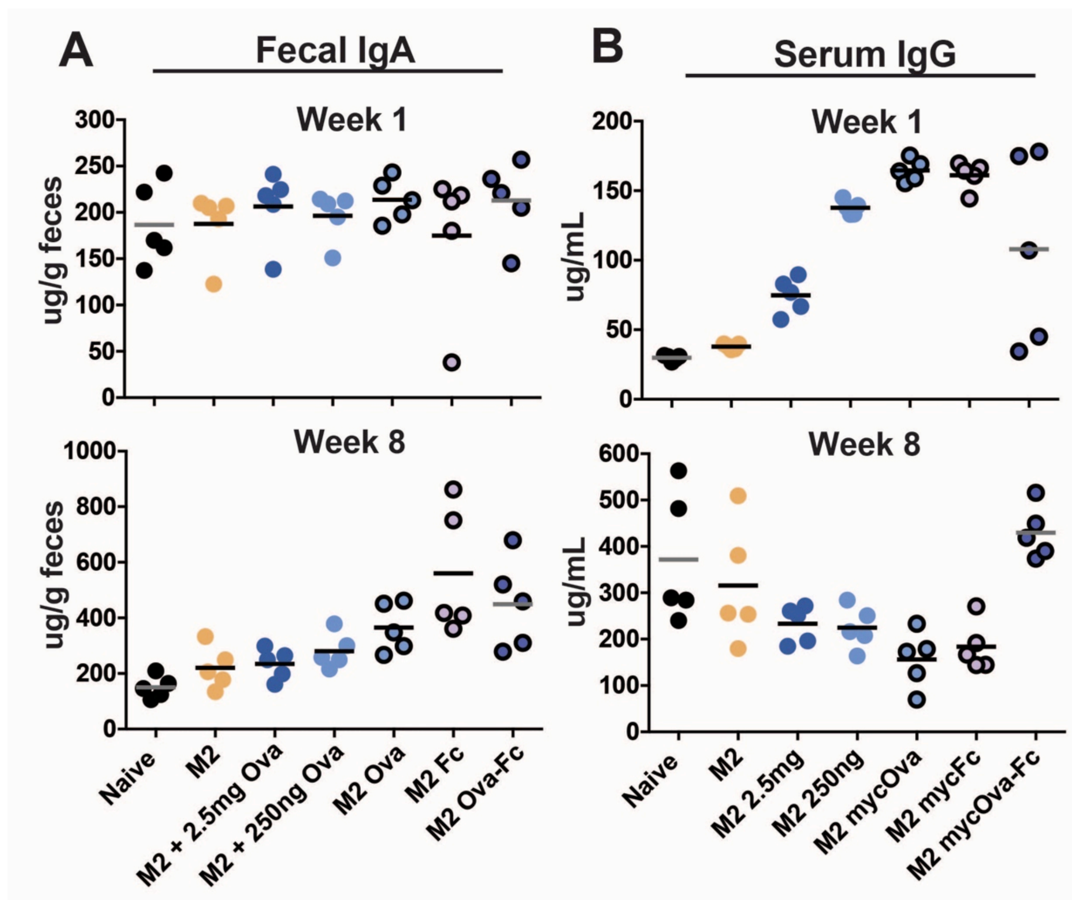


3533 **Fig 8.7 Antibody levels in mice gavaged with M2 expressing ovalbumin constructs**

3534 **do not differ from control mice**

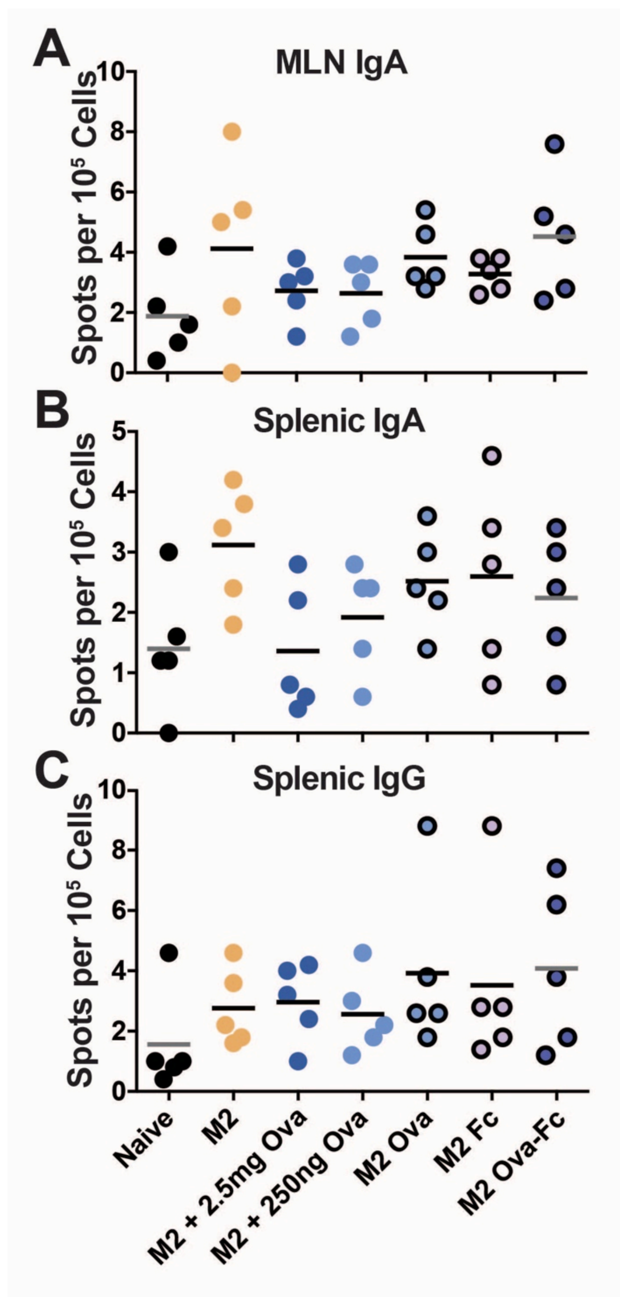
3535 Total fecal IgA (A) and serum IgG (B) levels were measured by ELISA in samples

3536 collected on weeks 1 and 8 after 2 and 8 total gavages, respectively. Plots each depict n =

3537 5 mice per group in one independent experiment.

3538

3539



3540

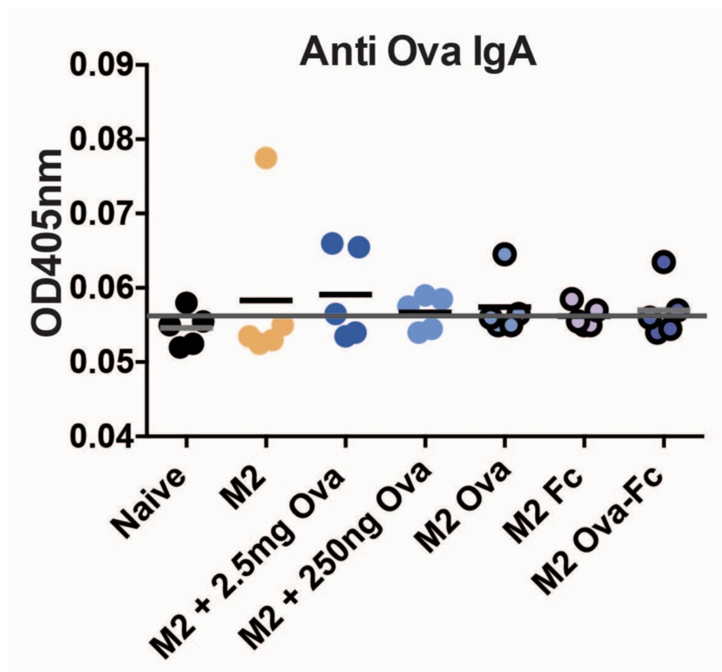
3541 **Fig 8.8 Antibody secreting cell numbers in mice gavaged with M2 transformed to**

3542 **express ovalbumin constructs do not differ from naïve mice**

3543 ELISPOT of mesenteric lymph nodes (MLN) (A), and spleens (B, C) collected at harvest

3544 after 8 total gavages shows high variability in the numbers of IgA and IgG secreting cells

3545 per 10^5 total cells and no detectable differences among groups (n = 5 mice per group).



3546

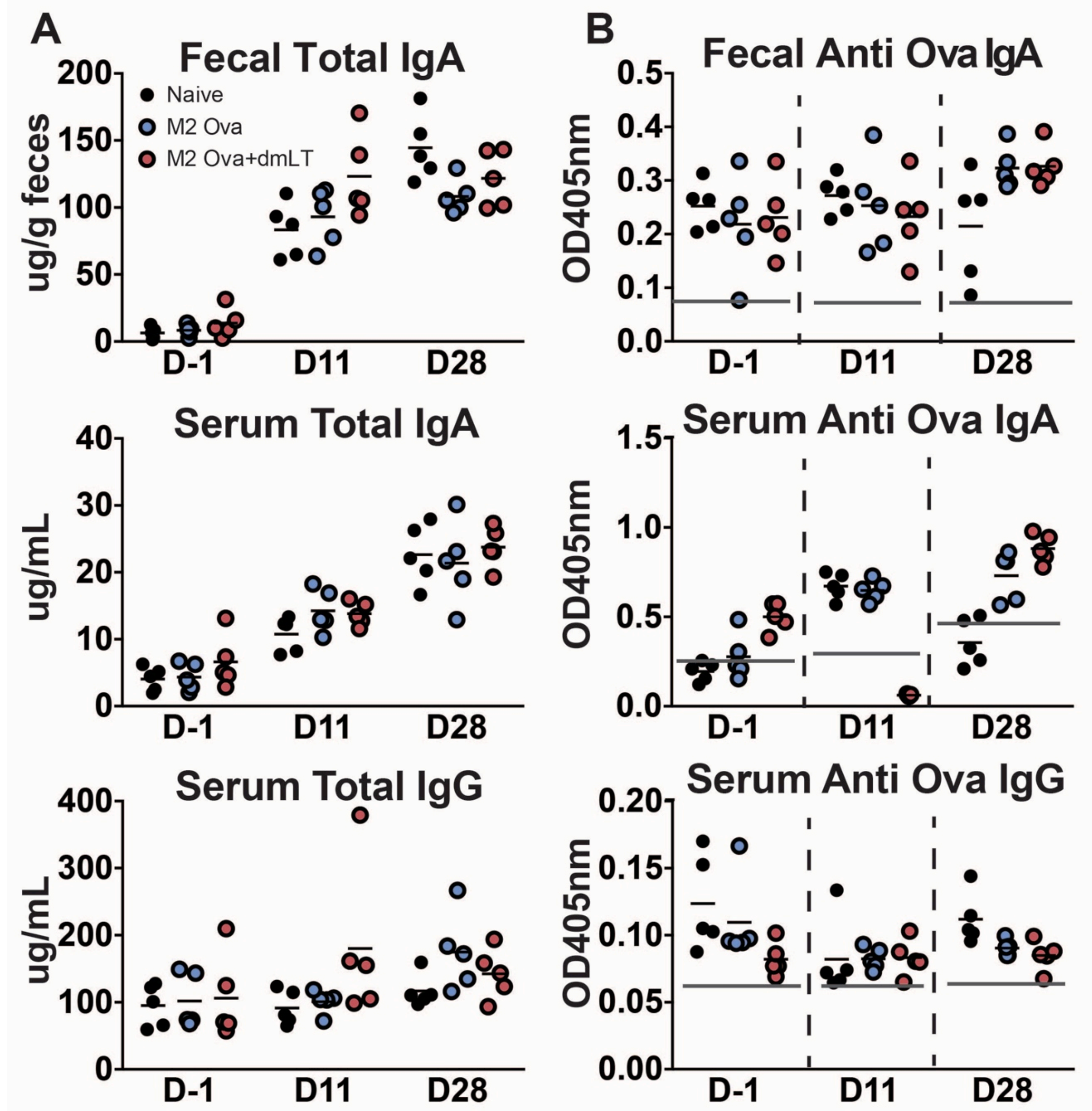
3547 **Fig 8.9 Ovalbumin-reactive antibody levels are below detectable limits**

3548 OD405nm values as determined by ELISA of serum collected at harvest indicate no

3549 difference in levels of ovalbumin-reactive IgA antibody, with all groups showing

3550 OD405nm values at the limit of detection (gray line, as determined by OD405nm values

3551 of blank wells) (n = 5 mice per group in one independent experiment).



3552

3553 **Fig 8.10 Antibody levels induced by M2 expressing ovalbumin constructs in**

3554 **conjunction with mucosal dmLT adjuvant do not differ from control groups**

3555 (A) Total antibody levels in serum and fecal samples were measured by ELISA on days -

3556 1, 11, and 28. Mice were gavaged with vehicle (Naïve, black circles), M2 expressing

3557 ovalbumin (M2 Ova, enclosed blue circles), or dmLT mixed with M2 expressing

3558 ovalbumin (M2 Ova + dmLT, enclosed pink circles). (B) OD405nm values as determined

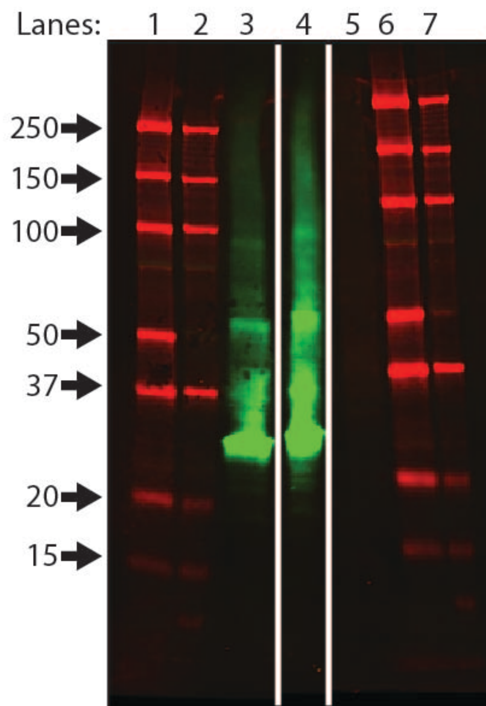
3559 by ELISA indicate no significant differences in levels of ovalbumin-reactive IgA or IgG

3560 antibody, with most values at background levels (depicted by gray lines, as determined

3561 by OD405nm values of blank wells at each time point) (n = 5 mice per group in one

3562 independent experiment).

3563



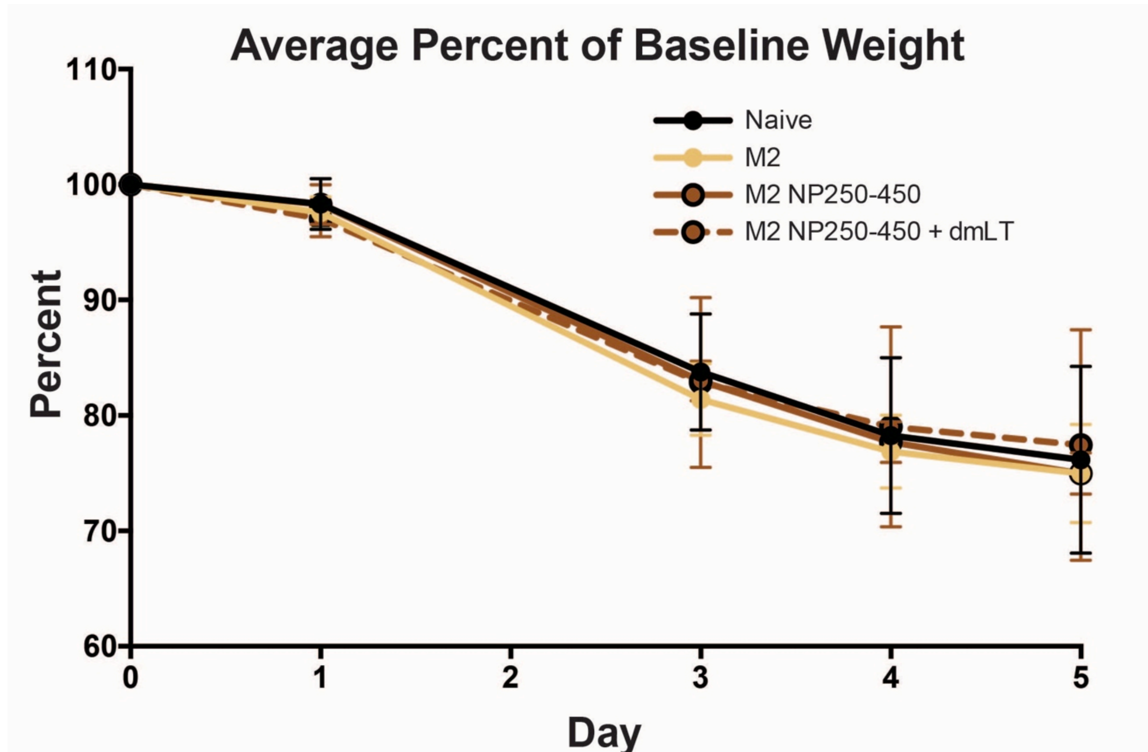
3564

3565 **Fig 8.11 Transformed M2 is able to express NP250-450**

3566 *S. boulardii* M2 was transformed with plasmid encoding myc-tagged NP250-450 and
3567 grown to saturation in selective media. Whole yeast lysate of transformed M2 (Lane 3)
3568 was run on a protein gel along with the Kaleidoscope (Lanes 1, 6) and WesternC (Lanes
3569 2, 7) standards. Lysates from transformed *S. cerevisiae* W303 (Lane 4) and
3570 untransformed M2 (Lane 5) were run as controls. Primary anti myc and secondary anti
3571 mouse IRDye-conjugated antibodies and an Odyssey LiCor Infrared imager were used
3572 for detection. Bands corresponding to the approximate 25kDa weight predicted for
3573 NP250-450 show successful expression in transformed M2 and W303 and no background
3574 in the untransformed negative control M2 (lanes are from the same original blot testing
3575 multiple additional constructs).

3576

3577



3578

3579 **Fig 8.12 M2 NP250-450 + dmLT vaccination fails to protect mice from lethal**

3580 **influenza challenge**

3581 Mice vaccinated with either vehicle (Naïve), 10^8 CFU M2, 10^8 CFU M2 expressing

3582 NP250-450 (M2 NP250-450), or 25 µg dmLT admixed with 10^8 CFU M2 expressing

3583 NP250-450 (M2 NP250-450 +dmLT) were weighed and challenged intranasally with a

3584 lethal strain of influenza A virus (A/Puerto Rico/8/1934(H1N1)) on day 0. Average

3585 mouse weight in each group is plotted as a percentage of baseline weight. Mice reached

3586 20% weight loss by day 5 and were euthanized. No differences in percent weight loss

3587 were observed between groups (n= 10 mice per group, error bars depict standard error of

3588 the mean).

3589

3590 **Table 8.1 Summary of Pilot Vaccination Experiments**

Experiment	Gavage Schedule	Antigen	Adjuvant	Relevant Figures
Ova	A	Soluble ovalbumin (mixed with untransformed M2)	M2	8.2A, 8.4-8.5
Ova-Fc	A	Ova and Ova-Fc expressed by M2	Fc	8.2A, 8.4, 8.6-8.9
Ova dmLT	B	Ova expressed by M2	dmLT	8.2B, 8.20
NP250-450	C	NP250-450 expressed by M2	dmLT	8.2C, 8.21, 8.22

3591
3592

3593 # 9) Discussion

3594 At the time the research presented in this manuscript began, *S. boulardii* was
3595 relatively undeveloped as a potential vaccine delivery system. Previous research had
3596 focused on discovering mechanisms of action of WT *S. boulardii* as an untransformed
3597 probiotic therapy in the context of numerous gastrointestinal diseases. Indeed, many
3598 studies have identified effects of *S. boulardii* on host cells and other microbes either *in*
3599 *vitro* or in the context of infectious or inflammatory disease models, such as *C. difficile*
3600 infection, db/db mouse models of diabetes and obesity, and others [132,171,274] (Tables 4.2
3601 and 4.4).

3602 However, there were few detailed studies of the localization and
3603 immunomodulation of *S. boulardii* in the healthy intestine and no analysis of how *S.*
3604 *boulardii* might contact antigen sampling and immune cells in healthy adult mice. One
3605 early study detected uptake of an *S. cerevisiae* strain into M cells in an ileal loop model in
3606 mini pigs [517]; however, the direct administration of yeast into the ileal loop in this model
3607 is not reflective of vaccination conditions where *S. boulardii* is administered orally and
3608 must transit through the entire intestine. Our own studies (Figs 6.6 and 7.8) demonstrate
3609 that only a small minority of the initial *S. boulardii* inoculum can be found in association
3610 with Peyer's patches in mice. Extensive histological analysis furthermore was unable to
3611 confirm uptake of *S. boulardii* across M cells into Peyer's patches, suggesting that
3612 although some *S. boulardii* is able to reach antigen sampling sites, uptake of intact yeast
3613 is a very low frequency event. It is thus likely that pilot vaccination studies (Chapter 8)
3614 were impeded by very limited exposure of immune cells to antigens and adjuvants
3615 expressed by transformed *S. boulardii*. These results, in addition to data showing that *S.*

boulardii has limited effects on antibody production and MLN transcript expression in the healthy adult mouse (Chapter 6), have several clear implications for further optimization of *S. boulardii*-based oral vaccines.

a) M cell targeting and antigen secretion may aid delivery of heterologous vaccine antigens to intestinal Peyer's patches

M cell targeting has been suggested as a means to increase efficiency of vaccine antigen delivery[482,483] and may be especially useful in prolonging contact between *S. boulardii* and M cells. Given the availability of auxotrophic mutant strains of *S. boulardii* (Chapter 7, also [332,518]), it is now possible to easily transform *S. boulardii* without antibiotic selection and enable heterologous protein expression. Experiments presented in Chapters 7 and 8 used transformed auxotrophic *S. boulardii* to express soluble heterologous proteins. Recent work has demonstrated that the yeast agglutinin receptor genes *AGA1* and *AGA2*, widely used for the generation of yeast display libraries on the *S. cerevisiae* cell wall [519], can similarly be used for expression of heterologous protein in the *S. boulardii* cell wall [316]. Use of this system to express M cell ligands within the cell wall may thus facilitate *S. boulardii*-M cell association of those yeast able to reach Peyer's patches. Indeed, expression of M cell ligands on the surface of probiotic bacteria has been proposed for experiments testing these strains for oral delivery of recombinant therapeutics [482,483]. Secretion of vaccine antigens, which has been achieved in *S. boulardii* using the alpha mating factor secretion leader sequence [331,520] and orthologous signal sequences of the heterologous protein being expressed [518], may furthermore allow for increased local concentrations of antigen available for uptake by M cells.

3639 However, the particular M cell ligands and vaccine antigens to use in this system
3640 should be carefully selected. A few groups have used antigen fusion to M cell ligands in
3641 oral vaccination experiments and found different effects on the induction of protective
3642 mucosal immunity. For example, one group found that fusion of antigen to Co1, which
3643 ligates the complement C5a receptor of M cells, increased mucosal responses to the
3644 dengue virus envelope domain III (EDIII) [482,521]. EDIII-Co1 expressed and secreted from
3645 *S. cerevisiae* also showed the ability to bind M cells *in vitro* [522]. In contrast, other studies
3646 found that fusion of ovalbumin to the M cell ligand reovirus protein sigma one (pσ1) led
3647 to suppressed OVA-specific plasma and mucosal IgA levels, suggesting that direct
3648 targeting of M cells can facilitate development of tolerance depending on the chosen
3649 antigen and ligand [345,523]. A range of M cell ligands may thus need to be tested for
3650 expression in *S. boulardii*, and the potential for induction of tolerance rather than
3651 protective immunity should be carefully evaluated.
3652

3653 ### b) Potential tolerogenic factors must be evaluated for optimization of
3654 ### an *S. boulardii* vaccine delivery vector

3655 In addition to the low frequency of contact between *S. boulardii* and antigen
3656 sampling sites, a few other factors may have influenced the lack of antigen specific
3657 responses in pilot vaccination studies using ovalbumin. Long-term exposure to proteins in
3658 food is known to promote the development of tolerance specifically to those antigens.
3659 This tolerance is characterized by lack of responsiveness to antigen both locally and
3660 systemically [344]. It is possible that the rodent diet used in these experiments may have
3661 contained small amounts of ovalbumin through inclusion of porcine meat chow, although

3662 there is no direct evidence of inclusion of chicken ovalbumin or egg white
3663 (http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/
3664 ~edisp/ducm04_028021.pdf). Still, regular rodent chow has been shown in numerous
3665 experiments not to prevent sensitization to systemic immunization with ovalbumin [524,525].
3666 While oral vaccination with ovalbumin is clearly distinct from the systemic immunization
3667 used to sensitize mice in the above experiments, it is thus still possible to induce antigen-
3668 specific responses to ovalbumin even with potential pre exposure to antigen in food.
3669 Future experiments testing *S. boulardii* expression of ovalbumin could
3670 specifically evaluate the ability of *S. boulardii* to induce protective responses versus
3671 tolerance by first orally vaccinating and then systemically immunizing with antigen.
3672 Responses to systemic ovalbumin immunization in mice orally vaccinated with *S.*
3673 *boulardii* expressing ovalbumin relative to mice orally gavaged with either purified
3674 ovalbumin or PBS may indicate whether delivery of antigen by *S. boulardii* potentiates
3675 either tolerogenic or inflammatory, antigen specific responses. This system could also be
3676 used to test delivery of recombinant *S. boulardii* in the presence or absence of particular
3677 adjuvants. Still, given the potential risk that pre exposure to ovalbumin may increase the
3678 difficulty of overcoming tolerance to this particular antigen, future vaccination
3679 experiments may be more successful if more immunostimulatory antigens such as
3680 pathogen toxins are selected.
3681

3682 **c) *S. boulardii* itself does not serve as a sufficient adjuvant**

3683 Given the current data, it is clear that neither *S. boulardii* itself nor the Fc
3684 adjuvant were able to overcome tolerance and induce specific antibody responses to

3685 ovalbumin as a test antigen. Indeed, there were no increased total or antigen specific
3686 antibody responses in the M2 Ova mouse group relative to naïve mice (Figs 8.7, 8.9).
3687 Interestingly, these results stand in contrast to numerous studies demonstrating either
3688 whole *S. cerevisiae* or cell wall components to have immunostimulatory properties when
3689 administered subcutaneously or *in vitro* [335,339,526,527]. Similar *in vitro* studies have also
3690 shown *S. boulardii* to induce high levels of IL-1β, IL-10, IL-12, IL-6, and TNF-α from
3691 human monocyte-derived DCs [289]. However, the ability of yeast cell wall components to
3692 act as adjuvants within the intestine may be limited both by structural differences that
3693 limit contact with APCs as well as phenotypic differences of mucosal APCs relative to
3694 bone marrow- or monocyte-derived cells.

3695 Still, efficacy of the *S. cerevisiae*-based oral vaccine expressing the ApxII toxin of
3696 *Actinobacillus pleuropneumoniae* indicates the potential for oral *Saccharomyces*-based
3697 vaccines to induce antigen-specific, protective immune responses [342,343,528] . In this
3698 system, *S. cerevisiae* delivered a peptide that is itself capable of stimulating inflammatory
3699 responses and damaging macrophages, endothelial cells, and others [529,530]. Another
3700 reported case of *S. boulardii* stimulating increased antigen-specific antibody levels
3701 entailed co-administration of *S. boulardii* with the highly inflammatory *C. difficile* toxin
3702 A [132]. Thus it appears that *S. boulardii* is able to potentiate immune responses to at least
3703 some inflammatory antigens; however, this may not extend to induction of antigen-
3704 specific responses against the potentially tolerogenic ovalbumin used in pilot vaccine
3705 studies. Evaluation of *S. boulardii* in the healthy mucosa (Chapter 6) also clearly
3706 demonstrates that *S. boulardii* alone does not induce inflammatory responses. Ability to
3707 overcome tolerance to vaccine antigens administered by *S. boulardii* will thus require

inclusion of a highly effective mucosal adjuvant. In our studies, both the novel Fc adjuvant and the well studied *E. coli* double mutant heat labile toxin dmLT were tested, as discussed below (Table 9.1).

d) Use of the Fc fusion system as a mucosal adjuvant in probiotic yeast requires further optimization

Several factors may have prevented ability of the first tested adjuvant, the novel Fc fusion system, to induce antigen specific responses to ovalbumin. Efficacy of these antigen-Fc fusion systems in previous studies has been attributed to possible effects of increased half life [531]. However, it is unlikely that this would play a major role in the context of the gastrointestinal tract. Some stimulation has also been attributed to xenogenic effects. Indeed, one study observed greater $CD8^+$ T cell responses using the Fc portion of human IgG1 than with endogenous mouse IgG2a, although responses were still induced using the endogenous mouse sequence [502]. Other studies also suggest that increased efficacy is due to ligation of activating Fc receptors on antigen presenting cells [531] (Fig 8.1). Fusion of antigen directly to an Fc receptor ligand mimics antigen opsonization and promotes APC Fc receptor cross linking, antigen phagocytosis, and presentation to T cells [501,531].

In order for this novel adjuvant to successfully opsonize vaccine antigen in an *S. boulardii*-based delivery system, proper expression and folding of Fc within recombinant yeast is crucial. The native Fc portion of mouse IgG2a is composed of the constant regions of two separate heavy chains, held together by multiple disulfide bonds at the hinge region [532]. In *S. cerevisiae*, the primary pathway for disulfide bond formation

3731 occurs within the oxidative conditions of the endoplasmic reticulum (ER), dependent
3732 upon the Ero1 (ER oxidoreductin) and PDI proteins [533,534]. Cytosolic expression of Fc in
3733 pilot vaccine studies may thus have prevented disulfide bond formation. Although there
3734 are conflicting reports as to the effect of Fc fragment disulfide bond reduction on
3735 downstream functions depending on the particular Ig subclass and cell types involved [535],
3736 it seems likely that lack of disulfide bonds limited the ability of Fc-antigen fusion
3737 peptides to ligate APC receptors.

3738 Design of Fc constructs further optimized for expression in yeast may permit the
3739 ability of this novel adjuvant to target antigen to APCs upon reaching Peyer's patches.
3740 Secretion of Fc-antigen constructs using leader sequences as described above (see "M
3741 cell targeting and antigen secretion…") would direct Fc constructs to the yeast ER for
3742 folding and assembly with disulfide bond formation. Certain mutant forms of the alpha
3743 mating factor leader sequence have also been developed to significantly increase
3744 expression of IgG1 in *S. cerevisiae* [536]. Thus, antigen-Fc constructs may be directed for
3745 secretion as dimeric rather than monomeric chains and improve Fc receptor ligation.

3746 Newly designed Fc constructs should be tested for the ability to ligate Fc
3747 receptors and trigger the necessary downstream signaling pathways. Numerous
3748 approaches, including use of flow cytometry and *in vitro* reporter systems, have been
3749 developed to assess antibody dependent cell phagocytosis and antibody dependent cell
3750 cytotoxicity subsequent to Fc receptor ligation [537]. Use of such tests will be particularly
3751 important for each antigen-Fc fusion construct designed as fusion to various therapeutic
3752 molecules has been shown to affect Fc affinity for its receptors [531]. Still, the ability of Fc
3753 fusion constructs to serve as adjuvants in vaccine systems will be effective only once the

S. boulardii vaccine delivery system itself has been optimized to ensure that these novel antigen-adjuvant constructs are able to contact Peyer's patches and APCs.

e) Co-Administration of Alternative Heterologous Adjuvants May Promote Induction of Antigen Specific Responses and Modulate T Helper Phenotypes

Alternate adjuvants may be needed in conjunction with Fc-antigen fusion constructs to provide the danger signals necessary for induction of protective immunity. Use of the dmLT mucosal adjuvant as an alternative to Fc in pilot vaccine experiments, however, failed to overcome tolerance to ovalbumin or induce protective responses against influenza challenge (Figs 8.10, 8.12). This result is unexpected given numerous previous demonstrations that dmLT increases antigen specific responses [506,507,538,539]. In the case of vaccination with ovalbumin, this finding lends further weight to the possibility that mucosal immune cells were not sufficiently exposed to vaccine antigen to induce specific responses. Additionally, the severe and rapid deterioration in health observed upon challenge with a lethal influenza strain may have prevented the ability of mice vaccinated with NP250-450 and dmLT to mount protective responses in time to avoid disease. Optimization of *S. boulardii* to deliver increased antigen to immune induction sites in the intestine and the use of nonlethal influenza strains for challenge may thus enable detection of protective immune responses in future experiments.

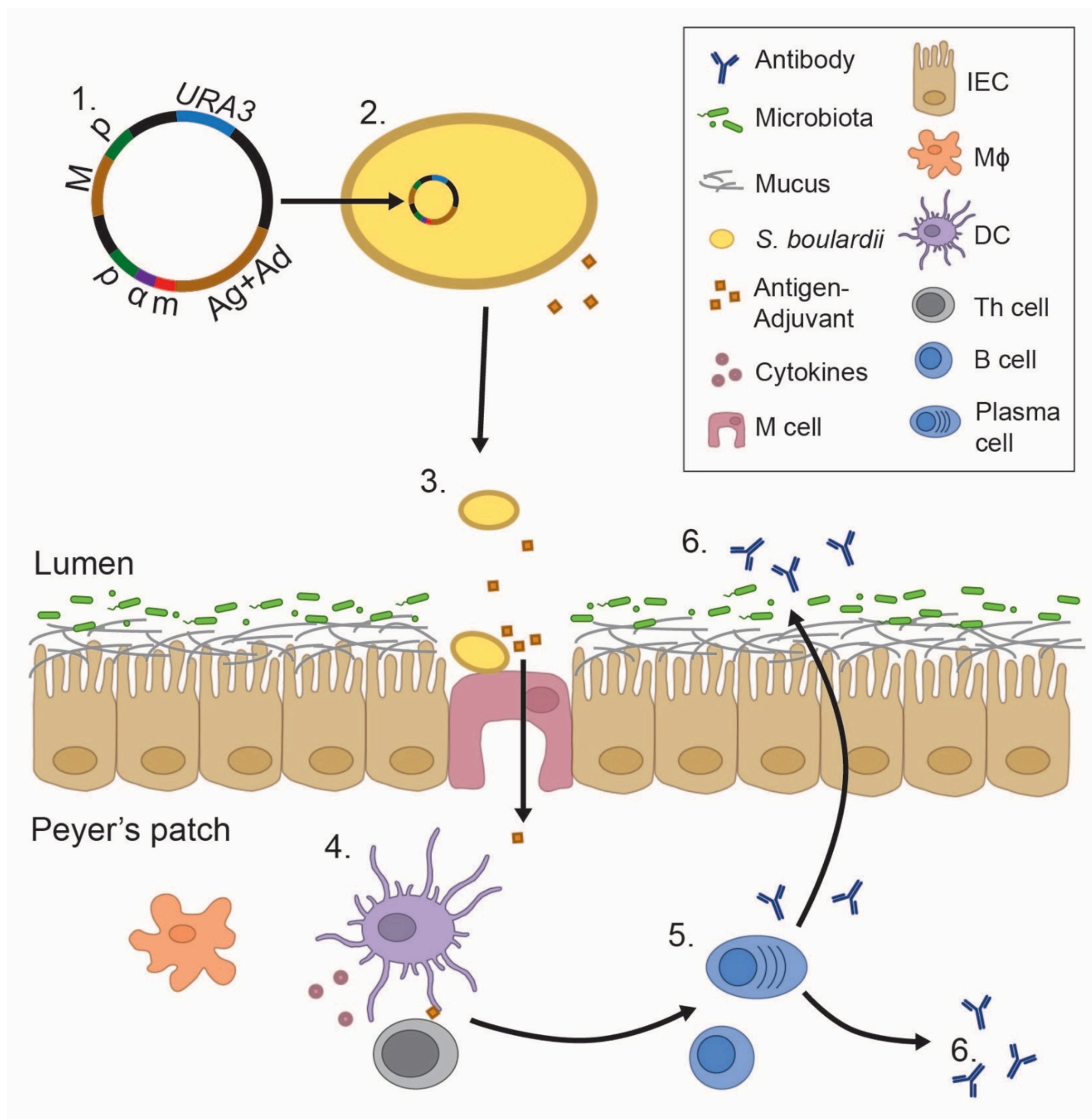
Still, alternate adjuvants expressed by *S. boulardii* itself may be necessary to improve vaccination efficiency and efficacy. One such potential adjuvant is the cytokine IL-12p70, a key inducer of inflammatory and T helper 1 (Th1) responses [353,540]. Indeed,

3777 heterologous expression of the inflammatory cytokine IL-12p70 has specifically been
3778 shown to induce protection in probiotic bacterium *Lactococcus lactis*-based oral vaccine
3779 models [541,542]. These studies found that co-administration of IL-12p70 alongside the
3780 *Leishmania* antigen LACK (*Leishmania* homologue of activated C kinase) was necessary
3781 to induce protective responses[541,542]. Production of functional IL-12p70 from *L. lactis*
3782 was increased when its two subunits, IL-12p35 and p40, were fused[543]. Furthermore,
3783 temporal and physical proximity of vaccine antigen and cytokine were key, with
3784 protection mediated only by co-expression of LACK and IL-12p70 within the same *L.*
3785 *lactis* strain[541]. No additional mucosal adjuvant was required in this system. Expression
3786 of IL-12p70 by *S. boulardii* may thus provide an alternative adjuvant to test in
3787 combination with antigen-Fc fusion constructs in order to promote protective, Th1
3788 responses.
3789

3790 **f) Summary**

3791 As a result of the experiments presented in these chapters, we now have a clearer
3792 illustration of the interactions of *S. boulardii* with the *in vivo* uninflamed gastrointestinal
3793 mucosa, possess the initial tools to develop *S. boulardii* as a vaccine delivery vector, and
3794 understand the next steps necessary to optimize both *S. boulardii* and vaccine adjuvants
3795 for this purpose (Fig 9.1). We have elucidated optimal means, in our hands, of
3796 transforming WT and auxotrophic strains of *S. boulardii*, permitting consistent
3797 transformation of these probiotic yeast (Chapter 5). Experiments in Chapters 7 and 8
3798 demonstrate that the *S. boulardii* M2 auxotrophic mutant can express a wide array of
3799 complex proteins, including ovalbumin, the novel adjuvant Fc, and a fragment of

3800 influenza nucleocapsid protein (NP). However, pilot vaccination experiments viewed in
3801 light of concurrent experiments characterizing *S. boulardii* within the healthy adult
3802 mouse intestine (Chapter 6), highlight the need for optimization both of *S. boulardii* itself
3803 and of antigen-adjuvant combinations to stimulate protective immune responses. Ongoing
3804 experiments aim to develop additional adjuvant constructs as well as targeted knockout
3805 yeast that will permit use of multiple auxotrophic markers and yield strains without the
3806 growth defects observed in the current *S. boulardii* $ura3^-$ auxotrophic strains. Together,
3807 these tools will enable the development of a novel means to efficiently and economically
3808 induce protective mucosal immunity using the probiotic yeast *S. boulardii*.
3809
3810



3811

3812 **Fig 9.1 New model of an *S. boulardii*-based oral vaccine delivery system**

3813 (1) Plasmids designed with the *URA3* auxotrophic marker take advantage of the new *S.*

3814 *boulardii* auxotrophic mutant strain M2 and eliminate the need for antibiotic selection, as

3815 described in Chapter 7. Use of the alpha mating factor secretion leader sequence (α) will

3816 induce secretion of vaccine antigen and adjuvant under the control of an inducible

3817 promoter (p) (as opposed to cytosolic expression under a constitutive promoter).

3818 Expression of M cell ligands using the *AGA1/2* gene expression system (M) will target *S.*

3819 *boulardii* for prolonged contact with these antigen sampling cells. (2) Although

3820 efficiency of WT *S. boulardii* transformation is low, possibly due to a thicker cell wall

3821 relative to *S. cerevisiae*, *S. boulardii* M2 can be efficiently transformed with LiOAc

3822 protocols, as described in Chapter 5. (3) *S. boulardii* appears to lack immunostimulatory

3823 capacity in the healthy intestine, as described in Chapter 6. Uptake of whole *S. boulardii*

3824 into M cells is a low frequency event in the healthy adult mouse intestine (described in

3825 Chapter 6), but M cell ligand expression and protein secretion systems may allow vaccine

3826 antigens and adjuvants to cross into Peyer's patches and (4) be taken up by DCs to induce

3827 protective immune responses. (5) B cell activation and plasma cell secretion of vaccine

3828 antigen-reactive antibody into (6) the intestinal lumen or periphery will mediate

3829 protection against challenge. Abbreviations: p, promoter; M, M cell ligand targeting

3830 system; α, alpha mating factor secretion leader sequence; m, myc tag; Ag, vaccine

3831 antigen; Ad, vaccine adjuvant; *URA3*, auxotrophic selection marker; DC, dendritic cell;

3832 IEC, intestinal epithelial cell; Mϕ, macrophage.

3833

3834 **Table 9.1 Tested and Proposed Adjuvants for Use with an *S. boulardii***

3835 **Vaccine Delivery Vector**

3836

Adjuvant	**Mechanisms of Action**	**Advantages**	**Disadvantages**
Tested Adjuvants			
Cytosolic Fc	• Fc receptor ligation	• Synthesis by *S. boulardii*	• Poor formation of dimeric complexes
dmLT	• cAMP-dependent and B subunit GM1 ganglioside receptor binding signals	• Well studied mucosal adjuvant	• Complex structure not yet optimized for expression in *S. boulardii*
Proposed Adjuvants			
Secreted Fc	• Fc receptor ligation	• Potential synthesis by *S. boulardii* • Ligation of Fc receptors on APCs	• May promote tolerance without coadministered danger signals
IL-12p70 Cytokine	• Co-stimulatory danger signal	• Potential synthesis by *S. boulardii* • Stimulate inflammatory responses and provide danger-associated signals	• May promote inflammatory damage to mucosa

3837
3838

3839 # 10) Appendix A: Abbreviations

3840 APC Antigen presenting cell
3841 CD Crohn's disease
3842 CDI *Clostridium difficile* infection
3843 CFSE Carboxyfluorescein succinimidyl ester
3844 DAMP Danger associated molecular pattern
3845 DC Dendritic cell
3846 dmLT *E. coli* heat labile toxin double mutant
3847 ETEC Enterotoxigenic *E. coli*
3848 FMT Fecal microbiota transplant
3849 GFP Green fluorescent protein
3850 HA Hemagglutinin
3851 IBD Inflammatory bowel disease
3852 IFN Interferon
3853 Ig Immunoglobulin
3854 IL Interleukin
3855 IVIG Intravenous immunoglobulin therapy
3856 LAB Lactic acid bacteria
3857 LGG *Lactobacillus rhamnosus* GG
3858 LPS Lipopolysaccharide
3859 M cell Microfold cell
3860 MLN Mesenteric lymph node
3861 NP Nucleocapsid protein
3862 NTCD Nontoxigenic *C. difficile*
3863 ORF Open reading frame
3864 OTU Operational taxonomic unit
3865 PP Peyer's patch
3866 PRR Pattern recognition receptor
3867 SCFA Short chain fatty acid
3868 SPF Specific pathogen free
3869 TcdA *C. difficile* toxin A
3870 TcdB *C. difficile* toxin B
3871 TER Transepithelial electrical resistance
3872 Th T helper cell
3873 TLR Toll-like receptor
3874 UC Ulcerative colitis
3875 UCDAI Ulcerative colitis disease activity index
3876 URA3 Orotidine-5'-phosphate decarboxylase
3877 WT Wild type
3878 ZO Zonula occludens

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