Synergistic Effects of Licorice Root and Walnut Leaf Extracts on Gastrointestinal Candidiasis, Inflammation and Gut Microbiota Composition in Mice

Hélène Authier,a Valérie Bardot,b Lucile Berthomier,b Bénédicte Bertrand,a Claude Blondeau,c Sophie Holowacz,c Agnès Costea

aGeroscience and Rejuvenation Research Center (RESTORE), UMR 1301-Inserm 5070-CNRS EFS, Université P. Sabatier, Toulouse, France
bPiLeJe Industrie, Saint-Bonnet-de-Rochefort, France
cPiLeJe Laboratoire, Paris, France

ABSTRACT Candida albicans is an opportunistic pathogen that causes gastrointestinal (GI) candidiasis closely associated with intestinal inflammation and dysbiosis. Drug resistance, side effects of available antifungal agents, and the high recurrence of candidiasis highlight the need for new treatments. We investigated the effects of hydroethanolic extracts of licorice root (LRE) and walnut leaf (WLE) on GI colonization by C. albicans, colon inflammation, and gut microbiota composition in C57BL/6 female mice. Oral administration of LRE and WLE alone or in combination once daily for 12 days before C. albicans infection and then for 5 days after infection significantly reduced the level of C. albicans in the feces of gastrointestinal infected mice as well as colonization of the GI tract, both extracts showing robust antifungal activity. Although total bacterial content was unaffected by the extracts (individually or combined), the abundance of protective bacteria, such as Bifidobacterium spp. and Faecalibacterium prausnitzii, increased with the combination, in contrast to that of certain pathobiont bacteria, which decreased. Interestingly, the combination induced a more robust decrease in the expression of proinflammatory genes than either extract alone. The anti-inflammatory activity of the combination was further supported by the reciprocal increase in the expression of anti-inflammatory cytokines and the significant decrease in enzymes involved in the synthesis of proinflammatory eicosanoids and oxidative stress. These findings suggest that LRE and WLE have synergistic effects and that the LRE/WLE combination could be a good candidate for limiting GI candidiasis and associated inflammation, likely by modulating the composition of the gut microbiota.

IMPORTANCE The adverse effects and emergence of resistance of currently available antifungals and the high recurrence of candidiasis prompt the need for alternative and complementary strategies. We demonstrated that oral administration of hydroethanolic extracts of licorice root (LRE) and walnut leaf (WLE) separately or in combination significantly reduced the colonization of the gastrointestinal (GI) tract by C. albicans, highlighting a robust antifungal activity of these plant extracts. Interestingly, our data indicate a correlation between LRE and WLE consumption, in particular the combination, and a shift within the gut microbiome toward a protective profile, a decrease in colonic inflammation and prooxidant enzymes, suggesting a synergistic effect. This study highlights the significant prebiotic potential of the LRE/WLE combination and suggests that the health benefits are due, at least in part, to their ability to modulate the gut microbiota, reduce inflammation and oxidative stress, and protect against opportunistic infection.

KEYWORDS candidiasis, gut inflammation, licorice, plant extract, prebiotic, walnut, microbiota
**Candida albicans** inhabit the gastrointestinal (GI) tract of most healthy individuals (1, 2). As a commensal member of the microbiota, the yeast is generally harmless, but it can become an opportunistic pathogen, particularly in individuals with impaired immunity (1). *C. albicans* is a major cause of infections worldwide; it commonly triggers superficial mucosal infections and may also, under favorable conditions, lead to potentially life-threatening deep tissue infections (1).

The GI tract is a key reservoir of *C. albicans*, and the fungus is well adapted to growth in the environment provided by the GI tract and to the changes that can take place within this, for example, following the use of antibiotics (2, 3). In addition, *C. albicans* has been associated with several GI diseases, such as celiac disease and inflammatory bowel diseases (3–7). *C. albicans* is thought to exacerbate inflammatory processes due to a sequence of mutually perpetuating events, including dysbiosis and low-grade inflammation in the gut that sustains the growth of the fungus while its excessive growth fosters further inflammation, increasing lesions and delaying healing (2, 3, 6). *C. albicans* is, therefore, considered to be involved in the pathogenesis of certain gastrointestinal diseases.

The emergence of drug resistance, the adverse effects of available antifungal agents, and the high recurrence rate of candidiasis have necessitated a search for new therapies. This has led to an increased interest in exploring the potential of plants for the treatment of fungal infections (8, 9).

The antifungal potential of licorice (*Glycyrrhiza glabra* L.) and walnut (*Juglans regia* L.) among other plants have been evaluated in several *in vitro* and *in vivo* studies. A propylene glycol extract of a dry powder of licorice roots inhibited the growth of *C. albicans in vitro* (10). Other studies investigated major phytochemical compounds extracted from licorice roots, such as the saponin glycyrrhizin, the aglycone of glycyrrhizin 18β-glycyrrhetinic acid, the chalcone licochalcone A, and the isoflavonoid glabridin (11–17). All these studies have contributed to demonstrating the antifungal potential of these compounds and thus of licorice root against *C. albicans* infections. For example, in an *in vivo* study with glycyrrhizin, mice were inoculated with *C. albicans* at lethal doses with or without previous administration of glycyrrhizin at the dose of 0.5 mg/kg/day for 15 to 20 days. Prior administration of glycyrrhizin decreased the mortality rate from 100% to 65%. Mean survival time increased from 7 to 11 days and symptom severity decreased (11).

There are fewer studies on the antifungal effects of a walnut leaf. The antifungal activity of walnut has been reported in a few *in vitro* studies evaluating different types of extracts. A hydromethanolic extract of walnut leaf was found to be the most effective of the plant extracts tested *in vitro* against *C. albicans* and other *Candida* species (18), confirming previous observations in studies investigating methanol, ethyl acetate, diluted acetone (19), and hydroethanolic extracts (20). It should be noted that in a study assessing aqueous extracts of different walnut leaf cultivars, no effect was observed on the tested fungi (*C. albicans* and *C. neoformans*) and Gram-negative bacteria species (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*). Only the growth of Gram-positive bacteria (*Bacillus cereus*, *B. subtilis*, and *Staphylococcus aureus*) was inhibited by these extracts (21).

In addition to their direct effects on *C. albicans* and their immunomodulatory properties, plants and their secondary metabolites have been shown to have prebiotic effects (22–27). These could be of interest in the context of GI candidiasis and other GI diseases given the demonstrated links between these diseases, *Candida*, inflammation, and dysbiosis. Among plant-derived compounds, phenolics, which encompass structural variants of flavonoids, hydroxybenzoic acids, hydroxycinnamic acids, coumarins, stilbenes, ellagitannins, and lignans can modify the composition of the gut microbiota (23, 24). The prebiotic potential of licorice root extracts has been evidenced *in vitro* (24, 25). Recent findings suggest that licorice (*Glycyrrhiza uralensis* Fisch.) could correct overall gut microbial dysbiosis and fecal metabolic disorders associated with CPT-11-induced colitis in mice (26). Compounds known to be present in walnut leaves, e.g., hydroxycinnamic acids and flavonoids, have also been reported to modulate gut microbiota composition (23, 24, 27).
Although many studies showed the in vitro antifungal effect against Candida sp. of licorice root extract and compounds (10, 14–16), only a few in vivo studies demonstrate its effect on candidiasis (11, 12, 17). Concerning walnut leaf, its antimicrobial effect was only demonstrated in in vitro assays (18–21). The objective of this study was to evaluate in vivo antifungal effects of specific hydroethanolic extracts of a walnut leaf (walnut leaf extract [WLE]) and licorice root (licorice root extract [LRE]), both separately and particularly in combination, in mice with GI candidiasis with the intention also to investigate whether the observed effects could involve anti-inflammatory activity and modulation of gut microbiota.

RESULTS

Phytochemical analysis of LRE revealed the presence of glycyrrhizin and several other compounds. High-performance thin-layer chromatography (HPTLC) analyses identified both glycyrrhizin acid (glycyrrhizin) and formononetin in LRE (Fig. 1). Ultra-high-performance liquid-chromatography–tandem mass spectrometry (UHPLC-MS) analysis (Fig. 2 and Table 1) confirmed the presence of glycyrrhizic acid (Fig. 2, peak 13) in LRE and identified enoxolone (glycyrrhetinic acid; peak 23). Various other acids, including citric acid (peak 3) and p-hydroxy benzyl malonic acid (peak 4) were also identified as well as flavonoids, such as liquiritin apioside and isoliquiritin apioside (peaks 6 and 6'), licuroside (peak 8), isoviolanthin (peak 5), 3-hydroxyglabrol (peak 21), and glabrol (peak 22).

LRE and WLE effectively reduced fecal colonization and gastrointestinal C. albicans infection in mice. To characterize the efficacy of plant extracts on the outcome of GI candidiasis, we evaluated the susceptibility of mice to Candida GI infection after oral administration of LRE and WLE separately or in combination (Fig. 3A).
We first evaluated the number of viable *Candida* in the stool that reflects both the colonization of the GI tract and the spontaneous yeast elimination following *Candida* oral administration. In accordance with a longer delay between *Candida* inoculation in mice and the day of the analysis of the fungal load in the stool, the number of viable yeast at day 5 compared to day 3 and day 4 postinfection was decreased (Fig. 3B).

When the two extracts were administered separately, although WLE tended to decrease the number of viable *C. albicans* in the feces from day 3 to day 4, only LRE achieved a significant decrease from day 3 to 5 postinfection. Interestingly, when the two plant extracts were administered together the number of viable *C. albicans* in the feces was substantially decreased from day 3 to 5 postinfection (Fig. 3B). The *C. albicans* loads in the esophagus, cecum, and colon at day 5 were significantly diminished in mice treated with LRE or WLE separately or in combination (Fig. 3C). Although WLE had no impact on the amount of *Candida* in the stool, the *Candida* colonization of the esophagus, cecum, and colon were significantly reduced at day 5 postinfection (Fig. 3C). Altogether, these results demonstrate that oral administration of LRE and/or WLE favors the clearance of *C. albicans* throughout the GI tract.

Oral administration of LRE, WLE separately or in combination influenced the composition of the colonic mucosa-associated microbiota in mice subjected to GI candidiasis. We evaluated the composition of colonic mucosa-associated bacteria in mice subjected to GI candidiasis that was treated with LRE, WLE, or the combination. Although the content of colonic mucosa-associated bacteria as a whole and that of the phylum Firmicutes were unaffected by LRE and WLE administered separately or in combination (Fig. 4A), the abundance of protective bacteria such as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* increased after administration of the two extracts combined. In line with this observation, the administration of LRE and WLE in combination tended to increase the content of *Lactobacillus* spp and *L. murinus*, which is described as a key beneficial bacteria for the health of the intestinal mucosa (28, 29).
| No. | t_R  | Compound                               | Formula       | Mass      | Ion m/z M-H theoretical | M-H (MS) | M-H (MS/MS) | Reference                        | M-H standard or ref (MS/MS) |
|-----|------|----------------------------------------|---------------|-----------|--------------------------|----------|-------------|----------------------------------|-------------------------------|
| 1   | 3.2  | Glucose                                | C\(_6\)H\(_{12}\)O\(_6\) | 180.06339 | 179.055                | (179)59/71 | 89/113/101/113/85 | (51) | (179)59/111/91/113/89/101/113/85 |
| 2   | 4.37 | Sucrose                                | C\(_{12}\)H\(_{22}\)O\(_{11}\) | 324.11621 | 341.1088               | (341)98/111 | 119/117/13/113 | (52) | (341)179/101/119/79/13/101/116/143/131 |
| 3   | 6.71 | Citric acid                            | C\(_6\)H\(_8\)O\(_7\) | 192.0270  | 191.0186               | (191)111/87/129/51/113/173 | (53) | (191)111/87/129/51/113/173 |
| 4   | 13.38| HBMA (p-hydroxybenzylmalonic acid)     | C\(_{27}\)H\(_{30}\)O\(_{14}\) | 578.16356 | 577.1566               | (577)111/87/129/51/113/173 | (54, 55) | fragmentation | (54) |
| 6   | 17.90| Succrose                                | C\(_{12}\)H\(_{22}\)O\(_{11}\) | 324.11621 | 341.1088               | (341)98/111 | 119/117/13/113 | (52) | (341)179/101/119/79/13/101/116/143/131 |
| 7   | 18.1  | Liquiritin apioside or isoliquiritin    | C\(_{26}\)H\(_{30}\)O\(_{13}\) | 550.16864 | 549.1614               | (549)255/135 | 119/255/113/148 | (53, 56) | for fragmentation | (53) |
| 8   | 33.68| Licoricesaponin J2                     | C\(_{26}\)H\(_{30}\)O\(_{13}\) | 550.16864 | 549.1614               | (549)255/135 | 119/255/113/148 | (53, 56) | for fragmentation | (53) |
| 9   | 41.56| Licoricesaponin A3                     | C\(_{15}\)H\(_{12}\)O\(_4\) | 254.05791 | 253.0506               | (253)117/135 | 255/113/153/148 | (53, 57) | for fragmentation | (53) |
| 10  | 43.46| Glycyrrhizin (glycyrrhizic acid)       | C\(_{42}\)H\(_{62}\)O\(_{16}\) | 822.40379 | 821.3965               | (822)113/193/85/71/72/75 | 99/175/289 | (61) | (822)761/647/539/351/333/289 |
| 11  | 44.03| Enoxolone                               | C\(_{30}\)H\(_{46}\)O\(_4\) | 470.33961 | 469.3323               | (469)351/113 | 781/776/775/704/661 | (54) | 805/779/647/539/351/333/289 |
| 12  | 46.48| Glycyrrhizin (glycyrrhizic acid)       | C\(_{42}\)H\(_{62}\)O\(_{16}\) | 822.40379 | 821.3965               | (822)113/193/85/71/72/75 | 99/175/289 | (61) | (822)761/647/539/351/333/289 |
| 13  | 46.96| Formononetin                            | C\(_{15}\)H\(_{12}\)O\(_4\) | 254.05791 | 253.0506               | (253)117/135 | 255/113/153/148 | (53, 57) | for fragmentation | (53) |
| 14  | 53.30| Glabridin                               | C\(_{25}\)H\(_{30}\)O\(_5\) | 408.19367 | 407.1864               | (407)235/113 | 781/776/775/704/661 | (54, 62) | for fragmentation | (54) |
| 15  | 54.81| Canzonol Y                              | C\(_{25}\)H\(_{30}\)O\(_5\) | 408.19367 | 407.1864               | (407)235/113 | 781/776/775/704/661 | (54, 62) | for fragmentation | (54) |
| 16  | 56.06| 3-hydroxyglabrol                        | C\(_{25}\)H\(_{30}\)O\(_5\) | 408.19367 | 407.1864               | (407)235/113 | 781/776/775/704/661 | (54, 64) | for fragmentation | (54) |
| 17  | 57.12| Glabrol                                 | C\(_{25}\)H\(_{30}\)O\(_5\) | 408.19367 | 407.1864               | (407)235/113 | 781/776/775/704/661 | (54, 64) | for fragmentation | (54) |
| 18  | 57.39| Enoxolone                               | C\(_{20}\)H\(_{20}\)O\(_4\) | 254.05791 | 253.0506               | (253)117/135 | 781/776/775/704/661 | (54, 64) | for fragmentation | (54) |

**TABLE 1** Compounds identified by liquid chromatography-mass spectrometry (LC-MS) in negative ionization mode.
At the same time, LRE and WLE separately or in combination significantly reduced Bacteroidetes and *Clostridium* spp. loads, which are often increased in dysbiosis. The content of Enterobacteriaceae was unaffected by the extracts (Fig. 4B). Thus, the LRE/WLE combination significantly shifted the composition of gut microbiota toward a protective profile.

**Oral administration of LRE and WLE separately or in combination reduced gut inflammation and improved the oxidative status of colonic tissues of *C. albicans*-infected mice.** To investigate the effect of LRE and WLE alone or in combination on colonic inflammation in mice infected with *C. albicans*, we assessed the expression of proinflammatory and anti-inflammatory markers in colonic tissues. Administration of the plant extracts separately or in combination decreased proinflammatory gene expression (*Il12p40*, *Tnfa*, *Il1b*, *Crr*, *Ccl2*). Interestingly, the combination of the two extracts induced a more robust decline in the expression of proinflammatory genes than either extract administered separately (Fig. 5A). These findings were corroborated by the reciprocal increase in the expression of *IL-10* and *TGF-β1* anti-inflammatory...
cytokines in colonic tissues of \textit{C. albicans}-infected mice that received the plant extracts (Fig. 5B).

Consistent with the decrease in proinflammatory markers induced, the LRE/WLE combination also decreased the mRNA expression of enzymes involved in the synthesis of proinflammatory eicosanoids (\textit{Ptgs2} [cyclooxygenase-2], \textit{Pges} [prostaglandin E synthase] and \textit{LTA4h} [LTB4 hydrolase], a critical enzyme for synthesis of the proinflammatory mediator LTB4) (Fig. 5C). The mRNA expression of enzymes involved in the synthesis of anti-inflammatory eicosanoids (\textit{Hpgds} [prostaglandin D synthase] and \textit{Alox15} [12/15-lipoxygenase]) was not affected by administration of the plant extracts (Fig. 5C).

Regarding the oxidative stress status of the colon, the mRNA expression of \textit{p47phox}, a cytosolic subunit of the NADPH oxidase complex, and the expression of inducible nitric oxide synthase (\textit{Nos2}), the activation of which is essential for the release of reactive oxygen species (ROS), were downregulated in response to the plant extracts with a stronger effect when the two extracts were combined (Fig. 5D). In accordance with this reduced oxidative status, the LRE/WLE combination shifted the balance between \textit{Nos2}...
Modulation of colonic inflammatory and oxidative status of *C. albicans*-infected mice treated with licorice root extract (LRE) and walnut leaf extract (WLE) alone or in combination. LRE and WLE alone or in combination, or vehicle were orally administered to mice (n = 10 per group) for 12 days. After this treatment, mice were orally infected with *C. albicans* and sacrificed 5 days later. Total RNAs isolated from the colon were subjected to the RT-PCR analysis using specific primer sets for (A) proinflammatory markers (*Il12p40* [Interleukin-12p40], *Tnfa* [Tumour Necrosis Factor alpha], *Il1b* [Interleukin-1 beta], *Crp* [C-reactive protein], *Ccl2* [C-C Motif Chemokine Ligand 2]), (B) for anti-inflammatory cytokines (*Il10* [Interleukin-10], *Tgfb1* [Transforming Growth Factor Beta 1], *Il1ra* [Interleukin-1 receptor antagonist]), (C) for enzymes involved in the production of pro- or anti-inflammatory eicosanoids (*Ptgs2* [cyclooxygenase-2], *Pges* [prostaglandin E synthase], *Lta4h* [LTB4 hydrolase], *Hpgds* [prostaglandin D synthase], *Alox15* [12/15-lipoxygenase]), (D) for pro-oxidant enzymes (*p47phox* [a cytosolic subunit of the NADPH oxidase complex], *Nos2* [inducible nitric oxide synthase]), and (E) for enzymes involved in anti-oxidant activities (*Arg1* [arginase-1], *Sod2* [superoxide dismutase], *Hemox-1* [hemoxygenase 1], *Nqo1* [NADPH quinone dehydrogenase 1], *Cat* [catalase-1]). Data are presented as means ± SEM. *, P ≤ 0.01; **, P ≤ 0.001; ***, P ≤ 0.005; ****, P ≤ 0.001 compared to vehicle. *, P ≤ 0.05; **, P ≤ 0.01 compared between treatments.
and arginase-1 toward the expression of arginase-1 (Fig. 5D and E). Moreover, although administration of the LRE/WLE combination slightly decreased Sod2 (superoxide dismutase) and Hemox-1 (hemooxygenase 1) concentrations, the combination significantly increased expression of the antioxidant enzymes Nqo1 (NADPH quinone dehydrogenase 1), Cat (catalase-1) and Arg1 (arginase-1) (Fig. 5E).

Altogether, these data highlight the anti-inflammatory and antioxidant potential of the LRE/WLE combination in the colon during gastrointestinal infection with C. albicans.

**DISCUSSION**

Although Candida spp. form part of the commensal microbiota in most individuals with a healthy immune system, variations in the local microenvironment, antibiotic treatment, or alterations in the immune system can favor dysbiosis and rapid proliferation of Candida, which can then become a pathogen (1, 2). The high incidence of fungal infections caused by Candida species and their increasing resistance to antimicrobial treatments, stimulate alternative approaches and new prophylactic therapies.

In the present study, we evaluated the effects of hydroethanolic extracts of licorice root (LRE) and walnut leaf (WLE), administered separately or in combination, on GI colonization by C. albicans, colon inflammation, and gut microbiota composition in mice. We observed that the level of C. albicans in the feces and colonization of the GI tract of infected mice treated with the plant extracts were significantly reduced. Interestingly, the two plant extracts administered together substantially decreased the number of viable C. albicans in the feces as well as the Candida burden in the esophagus, cecum, and colon, suggesting that the combination administered orally has synergistic effects and favors the clearance of C. albicans from the GI tract. In previous studies, licorice root extracts and specific compounds have consistently been shown to play a protective role against candidiasis in mice, owing to their ability to modulate the immune system and possibly to their prebiotic effects (11–15, 17, 24–26). Glycyrrhizin administered to mice inoculated with C. albicans at lethal doses decreased the mortality rate by 35%, increased mean survival time from 7 to 11 days, and decreased symptom severity (11). These effects were supported by the results of a study in MAIDS mice, which exhibit a 100 times greater susceptibility to C. albicans infection than wild-type mice, demonstrating the potential of glycyrrhizin to increase their resistance against C. albicans infection (12). In mice immunized with a C. albicans surface mannan extract in emulsion form, 1β-glycyrrhetinic acid (aglycone of glycyrrhizin) exerted a dominant Th1-immunological adjuvant effect (13). In vitro, this component also inhibited the growth of C. albicans isolated from patients with recurrent vulvovaginal candidiasis (14). Several additional studies have demonstrated the value of other licorice root compounds in fungal infections (13, 15–17). In vitro, the chalcone licochalcone A and the isoflavonoid glabridin showed antifungal activity against C. albicans (15). Licochalcone A had a significant inhibitory effect on biofilm formation, a key virulence factor, while both licochalcone A and glabridin inhibited the yeast-hyphae transition (15). Glabridin was also shown to induce C. albicans apoptosis via the caspase-independent route (16). Liquiritigenin, a flavonoid, increased the survival time of mice infected with C. albicans, this licorice root component protecting the mice against disseminated candidiasis by a CD4+ Th1 immune response (17).

Thus, the observed antifungal properties of the licorice root extract (LRE) evaluated in the present study could be due to the presence of flavonoids, such as glabridin and liquiritigenin, and to the presence of glycyrrhizic acid (and its derivative glycyrrhetinic acid), identified by UHPLC-MS analysis, and known to increase the resistance of mice to C. albicans infection (12, 13).

As we previously reported, the WLE tested contains several flavonoids, including quercetin, myricetin, kaempferol, and taxifolin derivatives as well as hydroxycinnamic acids (30). In a recent study, an extract of Trachyspermum ammi seeds enriched in rosmarinic acid-3-O-glucopyranoside, as well as kaempferol-(coumaroyl glucosyl)-rhamnoside and...
quercetin-3-O-galactoside, inhibited Candida in vitro (31). The anti-fungal effects of querce-
tin alone have been demonstrated in other in vitro studies (32, 33). It was reported that
the regulation of quorum sensing by quercetin, isolated from an edible lichen (Usnea long-
issima), could sensitize resistant C. albicans to fluconazole and thereby enhance the effi-
cacy of this drug. Quercetin enhanced the destruction of C. albicans NBC099 cells by fluco-
nazole and induced cell death. It was also found to strongly suppress the onset of
virulence-enhancing processes such as biofilm formation and hyphal development, as
well as phospholipase, proteinase, esterase, and hemolytic activities. The sensitization
was dependent on the farnesol response generated by quercetin, farnesol being a quo-
rum-sensing compound produced by C. albicans, that is known to regulate the expres-
sion of Candida virulence factors. In addition, taxifolin was identified as an inhibitor of the
transcriptional factors (Tec1 and Rfg1) inducing the hyphal growth responsible for the
invasiveness and virulence of C. albicans (34).

As microbiota composition of the GI tract influences the evolution of Candida from a
commensal to a pathogenic status and that licorice and walnut are described to have prebi-
otic effects (23–27, 35), we evaluated the effect of LRE and WLE on colonic microbiota in
mice with GI candidiasis. Our data indicate a correlation between LRE and WLE supplemen-
tation and a shift within the gut microbiome toward a protective profile. The relevant
increase in protective bacteria, such as Bifidobacterium spp. and Faecalibacterium prausnitzii,
known to have probiotic and anti-inflammatory properties (36–39), following oral adminis-
tration of the extracts in combination as well as the decrease in pathobionts, such as
Clostridium spp., support a synergic effect of the two extracts. These findings suggest that
regular supplementation may provide prebiotic benefits by modifying the composition and
diversity of the gut microbiota in such a way as to counteract Candida growth.

In line with its antimicrobial properties inhibiting Candida colonization of the GI tract
and the reorientation of the colonic mucosal microbiota toward protective bacteria, the
combination of the two plant extracts also alleviated colonic inflammation. We demon-
strated that the Candida burden was greatly diminished by the combination, a finding
consistent with the higher reduced colonic inflammation. In line with the anti-inflamma-
tory activity of the combination, several components of licorice, including glycyrrhizic
acid and isoliquiritigenin, which were detected in our extract, have been reported to
have anti-inflammatory, antioxidant and GI tract protective effects (40–43). Likewise, wal-
nut extract exhibits anti-inflammatory activities through nonchlorogenic and chlorogenic
acids known for their antioxidant and anti-inflammatory activities (30, 44–46).

Interestingly, the two plant extracts combined presented a synergistic anti-inflamma-
tory effect related to a greater reduction of proinflammatory cytokines and enzymes
involved in the synthesis of proinflammatory eicosanoids. Concomitantly, the two
plant extracts combined strongly induced the expression of anti-inflammatory cyto-
kines. Furthermore, this combination showed a stronger antioxidant potential resulting
from the downregulation of p47phox, Nos2, and the upregulation of antioxidant
enzymes in the colonic tissue of infected mice.

Altogether, our results suggest that the two plant extracts combined effectively
control GI candidiasis and the associated gut inflammation through their anti-inflamma-
tory and antioxidant properties, and their ability to modulate the composition of the
gut microbiota.

This study highlights the significant prebiotic potential of the LRE/WLE combination
and suggests that the health benefits of these plant extracts are due, at least in part, to,
their ability to modulate the gut microbiota, reduce inflammation, and oxidative stress,
and protect against opportunistic infection.

MATERIALS AND METHODS

Hydroethanolic extracts of licorice root and walnut leaf. In this study, we evaluated hydroetha-
nolic extracts of licorice (Glycyrrhiza glabra L) roots and walnut (Juglans regia L) leaves provided by
PiLeJe Laboratoire. The preparation and phytochemical analysis of the hydroethanolic extract of walnut
leaves was previously published (30). Briefly, a hydroethanolic extract of fresh walnut leaves (walnut leaf
extract [WLE]; PL-NOY-01; PiLeJe Laboratoire, France) was obtained according to a process similar to that
used for the licorice root extract described in detail below. In this previously published study,
chromatographic analyses had revealed the presence of various flavonoids (including quercetin, myricetin, kaempferol, and taxifolin derivatives) as well as hydroxycinnamic acids (including neochlorogenic acid).

**Preparation of the hydroethanolic licorice root extract.** Licorice (*Glycyrrhiza glabra* L.) roots were collected in Spain in November 2017. Fresh licorice roots were extracted by 20% to 70% (vol/vol) ethanolic leaching. The extracts were then mixed and concentrated under reduced pressure (100-pascal absolute pressure) at controlled temperature (35 to 45°C). Glycerol was then added to dilute the resulting extract to a final concentration of 5.2% (wt/wt) (referred to as licorice root extract [LRE]; PL-REG-01; PiLeJe Laboratoires, France).

**HPTLC analysis of the LRE.** Standards were diluted in ethanol 70% at a concentration of 0.4 mg/mL for glycyrrhizin acid and in methanol 0.1 mg/mL for formomononetin. The LRE without glycerol (1 mL) was diluted in 3 mL of a mixture of ethanol and water (70/30 vol/vol). The resultant solution was shaken and centrifuged for 5 min at 4400 rpm. The supernatant solution was transferred into individual vials and then submitted for HPTLC analysis. In addition, 1.8 g of ground licorice roots was extracted with 20 mL of ethanol and water (70/30 vol/vol). The resultant solution was sonicated for 10 min and centrifuged for 5 min at 4400 rpm. The supernatant solution was transferred into individual vials and then subjected to HPTLC analysis.

HPTLC analysis was performed on 200.0 × 100.0 mm silica gel 60 F 254 HPTLC glass plates (Merck, Germany). Standard solutions and samples were applied to the plate at 6.0 mm wide bands using CAMAG Automatic TLC sampler (ATS 4). The equipment comprised a CAMAG horizontal developing chamber, a TLC plate heater, a CAMAG Derivatizer Device, a CAMAG chromatogram immersion device, a CAMAG visualizer, and VisionCATS software. The general chromatography conditions are presented in Table 2.

**LC/MS analysis of the LRE.** Chromatographic analyses (UHPLC) were performed on an Ultimate 3000 RS LC UHPLC system (Thermo Fisher Scientific Inc., MA, USA) coupled to a binary pump (U3000 HPG-3400RS) and a diode array detector. Compounds were separated on an Uptisphere Strategy C18 column (25 × 4.6 mm; 5 μm; Interchim), which was controlled at 40°C. The mobile phase was a mixture of 0.1% (vol/vol) formic acid in water (phase A) and 0.1% (vol/vol) formic acid in acetonitrile (phase B). The gradient of phase A was 100% (0 min), 80% (10 min), 73% (35 min), 30% (50 min), 0% (55 min). The flow rate was 0.8 mL/min, and the injection volume was 5 μL. The UHPLC system was connected to a Q-Exactive Orbitrap (Thermo Fisher Scientific Inc., MA, USA) mass spectrometer, operated in negative and positive electrospray ionization mode. Source operating conditions: 3 kV spray voltage for negative mode and 3.5 kV spray voltage for positive mode; 320°C heated capillary temperature; 475°C auxiliary gas temperature; sheath, sweep, and auxiliary gas (nitrogen) flow rate 60, 18, and 4 arbitrary units, respectively; and collision cell voltage between 20 and 50 eV. Full scan data were obtained at a resolution of 35,000 whereas MS2 data were obtained at a resolution of 17,500. Data were processed using Xcalibur software (Thermo Fisher Scientific Inc., MA, USA).

Compounds present in the LRE were characterized according to their retention times, mass spectral data, and comparison with authentic standards when available or with published data.

**Murine model of gastrointestinal candidiasis.** All mouse experiments were performed according to protocols approved by the institutional ethics committee (CEEA122) and the French Ministry of Higher Education, Research, and Innovation (ESRI) under permit number 5412–2016051917498658;2016 to 2020 and renewed under permit number 23558–2020011016561848;2020 to 2025 in accordance with European legal and institutional guidelines (2010/63/UE) for the care and use of laboratory animals. Female C57BL/6 mice aged 8 weeks were purchased from Janvier Labs (France). LRE and WLE were administered orally, separately (2.5 g/kg) or in combination (1.25 + 1.25 g/kg), once daily for 12 days before *C. albicans* infection and then 5 days after infection. Control groups received only the vehicle (saline solution diluted with glycerol to the same extent as the extracts). Esophageal and GI candidiasis was established by intraesophageal administration of *C. albicans* at the rate of 50 × 10^6 blastospores in sterile saline solution per mouse, as described previously (47, 48). Ten mice were included in each
experimental group. Stools were collected daily from day 3 to day 5 after infection to quantify viable *C. albicans*. After 5 days of infection, the mice were sacrificed and the esophagus, cecum, and colon were aseptically removed to evaluate *C. albicans* colonization, microbiota composition, and inflammatory status.

**Preparation and quantification of viable *C. albicans* in stools.** The strain of *C. albicans* used throughout these experiments (sc-5314) was provided by the American Type Culture Collection (ATCC) and was maintained on Sabouraud dextrose agar (SDA; Bio-Rad, Hercules, CA, USA) plates containing gentamicin and chloramphenicol. Growth from an 18 to 24 h SDA culture of *C. albicans* was suspended in sterile saline solution (NaCl 0.9%) for mice infection (49, 50).

Stools were collected daily from day 3 to day 5 after infection, weighed, and mechanically homogenized in phosphate buffer saline (PBS). Serial dilutions of homogenates were plated on SDA plates containing gentamicin and chloramphenicol for the quantitative determination of the number of *C. albicans*. Plates were incubated at 37°C for 24 h and the number of colonies was counted to determine the colonies forming unit (CFU)/g of stool.

**Quantification of *C. albicans* in the gastrointestinal tract and microbiota analysis using real-time PCR.** The esophagus, cecum, and colon dissected from infected mice were crushed using lysing matrix tubes (MP Biomedicals, Illkirch, France). Tissue sample homogenates were resuspended in BLB lysis buffer (Roche Diagnostics, Meylan, France) for 20 min at room temperature and DNA was purified using a High Pure PCR Template Preparation kit (Roche). RT-PCR was performed on a Light Cycler 480 system using Light Cycler SYBR Green I Master Mix (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche). For amplicon detection, the Light Cycler DNA Analysis System using Light Cycler SYBR Green I Master (Roche).

To quantify the number of *Candida, C. albicans* cell suspensions were standardized at 10^6 cells/mL and serially diluted samples of genomic fungal DNA (range: 100 to 10^6 cells/mL) were used as external standards in each run. Cycle numbers of the logarithmic linear phase were plotted against the logarithm of the concentration of template DNA to evaluate the number of yeasts present in each tissue sample homogenate and normalized to the amount of genomic β-actin.

Semi-quantitative RT-PCR was performed with primers that amplify the genes encoding 16S rRNA from specific bacterial groups on DNA isolated from colonic mucosa to evaluate mucosa-associated bacterial colonization. Relative quantity was calculated and normalized to the amount of genomic β-actin.

**Gene expression analysis by reverse transcription and real-time PCR.** mRNA from colonic tissues were prepared and cDNA was synthesized according to the manufacturer’s recommendations (Total RNA MiniPreps super kit, BioBasic; Verso cDNA kit, Thermo Fisher Scientific). RT-PCR was performed on a Light Cycler 480 system with Light Cycler SYBR Green I Master Mix (Roche). Serially diluted samples of pooled cDNA were used as external standards in each run for the quantification. GAPDH was used as the housekeeping gene. The primers (Eurogentec), designed with the software Primer 3, were listed in Table 4.

**Statistical analysis.** GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) was used for graph preparation and statistical evaluation. Differences between groups were assessed using ANOVA, followed by a nonparametric Mann-Whitney test. Differences with *P* ≤ 0.05 were considered significant (*, *P* ≤ 0.05; **, *P* ≤ 0.01; ***, *P* ≤ 0.001; ****, *P* ≤ 0.0001). Data represent mean values ± standard error of the mean (SEM).

---

### Table 3 Primers used for gut microbiota analysis (68)

| Gene                        | 5′-3′ universal name | 5′-3′ sequence                  |
|-----------------------------|----------------------|---------------------------------|
| *Candida* spp. (69)         | sense                | TCGCATCGATGAAGACGCACG           |
|                            | antiseNSE            | TCTTTTCCCTCGCTATGATGCG          |
| *Clostridium* spp. (28)     | sense                | CCGTACTCAGCTAAAGACGG            |
|                            | antiseNSE            | AGTTCATTCGCGAACG               |
| *Bifidobacterium* spp. (28) | sense                | GGGTGTAAATGCCGGAATG             |
|                            | antiseNSE            | TAAGCGATGGACTCCACCG            |
| *Lactobacillus* spp. (28)   | sense                | AGCACTAGGAAGAATCTCCA           |
|                            | antiseNSE            | CACCCTACACATGGAAG              |
| Total bacteria (29)         | sense                | Eub338F                         |
|                            | antiseNSE            | Eub518R                         |
| *Bacteroidetes* (29)        | sense                | Bact934F                        |
|                            | antiseNSE            | Bact1060R                       |
| *Firmicutes* (29)           | sense                | Firm934F                        |
|                            | antiseNSE            | Firm1060R                       |
| *Enterobacteriaceae* (70)   | sense                | Uni515F                         |
|                            | antiseNSE            | Ent826R                         |
| *F. prausnitzii* (71)       | sense                | Fprau223F                       |
|                            | antiseNSE            | Fprau420R                       |
| *L. murinus/animalis* (72)  | sense                | ATGACCGAGATGATGTTTGA            |
|                            | antiseNSE            | ATGACCGAGATGATGTTG             |
| Genomic actin (73)          | sense                | TAAGCGATGGACTCCACCTG           |

March/April 2022 Volume 10 Issue 2 10.1128/spectrum.02355-21
TABLE 4 Primer sequences used in qRT-PCR

| Gene       | 5′-3′ Sequence | 3′-5′ Sequence |
|------------|----------------|----------------|
| Alox15     | sense          | GTCAGGAACCACAGGGAGG |
|            | antisense      | GTCAAGAGATCTGGTCGCCG |
| Arg1       | sense          | CGTGATATGCTGGTCCAGAG |
|            | antisense      | TGGCCTTTCCTGCTCCC |
| Cat        | sense          | ACATGTCTGGGACCTTCGG |
|            | antisense      | CAAGTGTTCAGGCTCATG |
| CCL2       | sense          | AGGACTCTGCTGGCTCTTCG |
|            | antisense      | TCTGACCCATTTCCTGTCG |
| Crp        | sense          | CGCACAGCTGTGCTTCCTC |
|            | antisense      | AGATGTGTGTTTTGACCTCA |
| Gapdh      | sense          | ACACATTGGGGGTAGGAACA |
|            | antisense      | AACTTGGCGATGTGGAAGG |
| Hemox-1    | sense          | CACGCATATACCCGCTACCT |
|            | antisense      | CCAGAGTGTCATGGAAGAAG |
| Hpgds      | sense          | GGAACGCTGGATGACTTCA |
|            | antisense      | TCCCGAGTAAGCTGTCGCA |
| Il10       | sense          | AGGCCGTCGATGATTTCT |
|            | antisense      | GCTCAGTGGGTCAGTATT |
| Il12p40    | sense          | AGGTCACTGCGACCAAAGG |
|            | antisense      | TGGTGGTGAGTGTCCTGCA |
| Il1ra      | sense          | GGCCTAGGTGCTCTGTCG |
|            | antisense      | TCAAGGGAGTCTCTGTCG |
| Il1b       | sense          | CAACCAACAGTGATATCTG |
|            | antisense      | GATCCACACTTCCTCCAGG |
| Lta4h      | sense          | GTTGACAGCTGAAACCAGAT |
|            | antisense      | CTCGCCCTAGTCCACAT |
| Nos2       | sense          | TCCCTGGACATTACGAGG |
|            | antisense      | ACAAGGCCTCTCCATG |
| Nqo1       | sense          | TTCTCTGTGCGGATGAGT |
|            | antisense      | GGCCTGCTGGAGAATAAG |
| Pges       | sense          | CCTAGGCCTGCTGCTGAC |
|            | antisense      | CAAGCTTTGTGTGACGACA |
| Ptgs2      | sense          | AGAAGGAAATGGCTGCAAG |
|            | antisense      | GCTGCGCTTCCAGTATGG |
| p47phox (Ncf1) | sense      | AGTGATGGGAGACATGGT |
|            | antisense      | ACCGAGTACAGGCAAAATG |
| Sod2       | sense          | GCCCCTGAGTGGTTGATA |
|            | antisense      | AGACAGCAGAGGCTCTACCA |
| Tgfb1      | sense          | AGGTTGGCCATTCCACTCC |
|            | antisense      | AGGGGGCTCTAAGGACAGTC |
| Tnfa       | sense          | AGCCGCCAGTGCTGTCCTT |
|            | antisense      | CTCCTTGGCAGAACTCAGG |

ACKNOWLEDGMENTS

This research was funded by PiLeJe Laboratoire, Paris, France. We thank Philippe Batigne (RESTORE UMR 1301-Inserm 5070-CNRS EFS Université P. Sabatier, Toulouse, France) for technical support in the animal studies.

Conceptualization and methodology: A.C., S.H.; formal analysis and investigation: H.A., V.B., L.B., B.B.; writing - original draft preparation: H.A., C.B.; writing - reviewing, and editing: H.A., C.B., A.C., S.H.

V.B., L.B., C.B., and S.H. are employees of PiLeJe and were involved in the design, investigation, writing of the manuscript, and decision to publish.

REFERENCES

1. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K. 2018. Candida albicans - Biology, molecular characterization, pathogenicity, and advances in diagnosis and control - An update. Microb Pathog 117: 128–138. https://doi.org/10.1016/j.micpath.2018.02.028.
2. Pérez JC. 2019. Candida albicans dwelling in the mammalian gut. Curr Opin Microbiol 52:41–46. https://doi.org/10.1016/j.mib.2019.04.007.
3. Kumamoto CA. 2011. Inflammation and gastrointestinal Candida colonization. Curr Opin Microbiol 14:386–391. https://doi.org/10.1016/j.mib.2011.07.015.
4. Poulin D. 2015. Candida albicans, plasticity and pathogenesis. Crit Rev Microbiol 41:208–217. https://doi.org/10.1080/1040841X.2013.813904.
5. Kowalska-Duplaga K, Krawczyk A, Sroka-Oleksiak A, Salamon D, Wędrzychowicz A, Fydek R, Gosiewski T. 2019. Dependence of colonization of the large
intestine by Candida on the treatment of Crohn’s disease. Pol J Microbiol 68:121–126. https://doi.org/10.21307/pjm-2019-014.
6. Li J, Chen D, Yu B, He J, Zheng P, Mao X, Yu J, Luo J, Tian G, Huang Z, Luo Y. 2018. Fungi in gastrointestinal tract of human and mice: from community to functions. Microb Ecol 75:821–829. https://doi.org/10.1007/s00248-017-1105-9.
7. Sokol H, Leducq V, Ashard H, Pham H-P, Jegou S, Landman C, Cohen D, Ligiouri G, Bourrier A, Nion-Larmorur I, Cosnes J, Siskin P, Langella P, Skurnik D, Richard ML, Beaugerie L. 2017. Fungal microbiota dysbiosis in IBD. Gut 66:1039–1049. https://doi.org/10.1136/gutjnl-2015-310746.
8. Zdia A, Bamba S, Yacouba A, Ouedraogo-Traore R, Guiguemdé RT. 2017. Ant-Candida albicans natural products, sources of new antifungal drugs: a review. J Mycol Med 27:19–1. https://doi.org/10.1016/j.jmmm.2016.10.002.
9. de Maia CMA, Pasetto S, Nonaka CFW, de Costa EMMB, Murata RM. 2021. Yeast-host interactions: Anadenanthera colubrina modulates virulence factors of C. albicans and inflammatory response in vitro. Front Pharmacol 12:269778. https://doi.org/10.3389/fphar.2021.629778.
10. de Oliveira JR, de Castro VC, das Graças Figueiredo Vilela P, Camargo SEA, Biao Xiao. 2013. Cytotoxicity of Brazilian plant extracts against oral microorganisms of interest to dentistry. BMC Complement Altern Med 13:208. https://doi.org/10.1186/1472-6882-13-208.
11. Guo N. 1991. Protective effect of glycyrrhizine in mice with systemic Candida albicans infection and its mechanism. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 38:380–383.
12. Utsunomiya T, Pasetto S, Nonaka CFW, de Costa EMMB, Murata RM. 2021. Yeast-host interactions: Anadenanthera colubrina modulates virulence factors of C. albicans and inflammatory response in vitro. Front Pharmacol 12:269778. https://doi.org/10.3389/fphar.2021.629778.

Licorice Root and Walnut Leaf Antifungal Synergy Microbiology Spectrum March/April 2022 Volume 10 Issue 2 10.1128/spectrum.02355-21

41. Peng F, Du Q, Peng C, Wang N, Tang H, Xie X, Shen J, Chen J. 2015. A review: the pharmacology of isoliquiritigenin. Phytother Res 29:89–97. https://doi.org/10.1002/ptr.5348.
42. Liu D, Huo X, Gao L, Zhang J, Ni JH, Cao L. 2018. NF-κB and Nrf2 pathways contribute to the protective effect of Licoricecone A on dextran sulphate sodium-induced ulcerative colitis in mice. Biomed Pharmacother 102:922–929. https://doi.org/10.1016/j.biopha.2018.03.130.
43. Zhao L, Chen X, Shao X, Wang Z, Du Y, Zhu C, Du W, Tang D, Ji S. 2021. Prenylated phenolic compounds from licorice (Glycyrrhiza uralensis) and their anti-inflammatory activity against osteoarthritis. Food Funct 13: 795–805. https://doi.org/10.1039/D1FO0359A.

44. Shin HS, Satush H, Bae M-J, Zhao Z, Ogihara W, Totsuka M, Shimizu M. 2015. Anti-inflammatory effect of chlorogenic acid on the IL-8 production in Caco-2 cells and the dextran sulphate sodium-induced colitis symptoms in C57BL/6 mice. Food Chem 168:167–175. https://doi.org/10.1016/j.foodchem.2014.06.100.

45. dos Santos MD, Almeida MC, Lopes NP, de Souza GEP. 2006. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. Biol Pharm Bull 29:2236–2240. https://doi.org/10.1248/bpb.29.2236.

46. Satoh Y, Itagaki S, Kurokawa T, Ogura J, Kobayashi M, Hirano T, Sugawara M, Ieki K. 2011. In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. Int J Pharm 403:136–138. https://doi.org/10.1016/j.ijpharm.2010.09.035.

47. Lefèvre L, Authier H, Stein S, Majorel C, Couderc B, Dardenne C, Eddine S, Guo D. 2013. An integrated exact mass spectrometric strategy for identification and chemical stand-ardization of licorice raw materials and dietary supplements using UHPLC-MS/MS. J Agric Food Chem 61:8062–8070. https://doi.org/10.1021/jf302954w.

48. Celano R, Docimo T, Piccinelli AL, Rizzo S, Cifuentes A, Herrero M. 2016. Metabolite profiling of licorice (Glycyrrhiza glabra) from different locations using comprehensive two-dimensional liquid chromatography coupled to diode array and tandem mass spectrometry detection. Anal Chim Acta 913:145–159. https://doi.org/10.1016/j.aca.2016.01.040.

49. MassBank of North America. https://mona.ehnlab.ucdavis.edu/spectra/display/PR100559. Accessed September 22, 2021.

50. MassBank of North America. https://mona.ehnlab.ucdavis.edu/spectra/display/PM019111. Accessed September 22, 2021.

51. Coste A, Dubourdeau M, Linas MD, Cassaing S, Lepert J-C, Balard P, Arnal J-F, Auwerx J, Pipy B. 2008. IL-13 attenuates gastrointestinal candidiasis in normal and immunode-ficient mice via peroxisome proliferator-activated receptor-gamma activation. J Immunol 180:4939–4947. https://doi.org/10.4049/jimmunol.180.7.4939.

52. Coste A, Dubourdeau M, Linas MD, Cassaing S, Lepert J-C, Balard P, Chalmeton S, Bernad J, Orti A. 2003. PPARgamma promotes mannose receptor gene expression in murine macrophages and contributes to the induction of this receptor by IL-13. Immunity 19: 329–339. https://doi.org/10.1016/S1074-7613(03)00229-2.

53. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleisher PR, Dubinsky M, Rotter JI, Wang HL, McGovern DPB, Brown GD, Underhill DM. 2012. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science 336: 1314–1317. https://doi.org/10.1126/science.1221789.