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# Interpreting probiotic and combination-therapy studies in *Blastocystis*

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**Abstract**
Interest in probiotic-based adjuncts to anti-protozoal therapy is justified, particularly where clinical responses to metronidazole are inconsistent. However, claims in the *Blastocystis* literature are frequently weakened by suboptimal design and reporting problems that make results difficult to interpret, reproduce, or translate. Here, we discuss a recently published Gut Pathogens study evaluating metronidazole, *Lactobacillus* probiotics, and combination therapy in vitro and in a mouse model. The core idea is plausible, but the paper illustrates recurring pitfalls that risk misleading readers: internal inconsistencies between the abstract and methods in dosing and concentrations, probiotic experiments without controls to separate parasite-specific effects from culture chemistry (vehicle and pH effects), reliance on microscopy-only burden estimates without molecular confirmation, and immunostaining results that appear internally contradictory and methodologically unclear. These issues matter because probiotic interventions can alter pH, redox status, nutrient availability, and immune status independently of any direct antiparasitic activity. Without appropriate controls, apparent "synergy" can be an artefact. We outline a minimum set of controls and reporting items that would make similar studies interpretable, including in vitro excipient- and pH-matched controls, uninfected intervention arms in vivo, blinded scoring, quantitative parasite burden as assessed by qPCR, and standard immunostaining controls. A community-wide emphasis on these basics would improve reproducibility, reduce overinterpretation, and accelerate progress toward genuine mechanistic understanding of *Blastocystis* in the gut ecosystem.

**Keywords** *Blastocystis*, probiotics, metronidazole, experimental design, reproducibility, immunohistochemistry, parasite burden

## A plausible hypothesis, but the purported evidence must be interpretable

Strategies for managing colonisation with *Blastocystis* may be worth exploring. Metronidazole remains widely used; yet treatment outcomes vary, and the clinical significance of *Blastocystis* detection remains contested. This is why careful experimental design matters: if a study is under-controlled or inconsistently reported, it not only undermines interpretations; it actively increases confusion in a field already prone to circular citations and overextended conclusions.

Under-reporting of experimental details and over-extension of limited datasets have predictable

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consequences: they inflate the perceived volatility of the field, impede meaningful synthesis across studies, and encourage premature clinical extrapolation in settings where parasite burden, subtype/strain, and confounding have not been resolved. Importantly, more interpretable approaches already exist in the *Blastocystis* literature. For example, antimicrobial susceptibility studies have used panels of isolates and clearly reported in vitro endpoints [1], while co-culture experiments have specified subtype and the bacterial strains tested, enabling comparison across laboratories [2]. Work exploring interactions between defined *Blastocystis* subtypes and gut microbial communities further illustrates the value of explicit culture status and quantitative readouts when making mechanistic inferences [3]. These studies are not perfect, but they demonstrate that clearer reporting and control sets are feasible in practice.

Baz Mohamed and colleagues report improved parasitological outcomes (reduced *Blastocystis* counts and viability in vitro, and reduced stool and intestinal cyst counts in infected mice) alongside host-associated readouts, including improved small- and large-intestinal H&E histopathology, increased IgA staining in intestinal sections, and reduced serum IL-1β, IL-6, and IFN-γ, with the largest changes reported in the metronidazole-plus-probiotic group [4]. At first, the overall message appears appealing. However, several features of the paper prevent a confident assessment of what was actually tested and what the results mean.

#### Internal inconsistencies that compromise reproducibility

The article contains substantial inconsistencies between the Abstract and the Methods with respect to key experimental parameters. In vitro, the Abstract reports metronidazole at 10 μg/mL and probiotics at 10^8 CFU/mL, whereas the Methods describe metronidazole at 120 μg/mL and probiotics at 10^9 CFU/mL [4]. In vivo, the Abstract reports metronidazole at 20 mg/kg, while the Methods state 120 μg/kg/day [4]. These slips change the biological plausibility of the results and make the work difficult to reproduce. Before debating mechanisms or clinical implications, the conditions that were actually used should be clarified.

#### In vitro probiotic experiments need controls for culture chemistry and formulation effects

The probiotics were delivered from a commercial sachet containing lyophilised organisms plus unknown excipients (non-active ingredients in the commercial preparation that can alter pH, osmolarity, or nutrient availability), reconstituted in water and added to Jones' medium with *Blastocystis* [4]. In this setting, "probiotic effect" can mean several different things:

- a direct effect of live *Lactobacillus* on *Blastocystis* (contact-dependent or via secreted metabolites).
- an indirect effect mediated by pH reduction, redox shifts, or nutrient competition.
- an excipient or osmolarity effect from the sachet formulation.
- an artefact of bacterial growth in a xenic system that changes microscopy-based counting and "viability" readouts.

Without specific controls, these effects cannot be differentiated and identified. As a minimum, studies of this type should include:

- a vehicle or excipient-only control (the sachet matrix without viable bacteria).
- a pH-matched control (culture medium adjusted to the same pH achieved in the probiotic condition).
- heat-killed bacteria controls to distinguish live biological activity from non-viable biomass effects.
- cell-free supernatant versus washed cells to separate secreted metabolites from cell-associated effects.

None of these controls are presented in the study [4]. As a result, reductions in counts and trypan blue-based "viability" cannot be interpreted as parasite-specific inhibition as they may also reflect non-specific environmental stress. This is especially important when the authors mention combination treatment being "superior" and imply enhanced efficacy beyond either agent, metronidazole (MTZ) and a *Lactobacillus* probiotic preparation, tested alone and in combination [4]. If synergy is claimed, it should be shown as synergy. A numerical improvement over monotherapy is not sufficient. Standard interaction analyses (for example, checkerboard designs and fractional inhibitory concentration approaches) exist precisely to prevent overcalling additive or confounded effects as synergy.

#### In vivo attribution is limited by missing arms and microscopy-only burden measures

In the in vivo part of the study, the design includes infected untreated and infected treated groups, plus an uninfected control [4]. The non-infected intervention arms (non-infected + metronidazole; non-infected + probiotics) is entirely missing. These arms matter because metronidazole and probiotics can alter intestinal inflammation and cytokine profiles independently of parasite clearance. Without them, it is difficult to attribute improvements in histology and cytokine profiles to antiparasitic activity rather than direct immunomodulation.

Parasite burden is assessed by microscopy of stool sediments and intestinal wash preparations [4]. Microscopy is useful but insufficiently specific on its own for "near

elimination" claims, particularly in complex intestinal samples. A molecular quantification (qPCR) targeting *Blastocystis* would provide an independent, quantitative measure of burden and help validate microscopy-based results. This is a straightforward, standardisable, and widely used control in contemporary parasitology and microbiome-adjacent research.

Blinding is also not described for parasite counts, histopathology scoring, or immunostaining assessment [4]. Given the subjective nature of these readouts, blinding should be explicitly stated and, ideally, paired with inter-rater reproducibility for histology and immunostaining.

### IgA staining appears internally contradictory and methodologically unclear

Reporting of IgA immunohistochemistry is difficult to reconcile across the text, tables, and figure legends. The Methods describe FITC-conjugated anti-mouse IgA antibodies [4], suggesting fluorescence detection, yet the figure legends describe H&E context and refer to "nonspecific IgA staining" in inflammatory infiltrates [4]. Additionally, Table 3 reports negative IgA expression in the control group, whereas the Results text states that the control shows strong IgA expression [4]. These contradictions undermine confidence in the IgA conclusions and by extension, in the mechanistic narrative that combination therapy "restores" mucosal immunity. Furthermore, in the aforementioned manuscript, in Figs. 4 and 5 the structures interpreted as *Blastocystis* are not supported by organism-specific staining or labelling; without a specific marker or molecular corroboration, morphology-based attribution in routine histology is not sufficiently specific to confidently assign these structures to *Blastocystis*.

For immunostaining intended to support mechanistic claims, a minimum control set is non-negotiable:

- no-primary antibody control.
- isotype control.
- known-positive tissue processed in parallel.
- clear description of imaging modality and quantification approach.

If these were performed, they should be reported. If they were not, IgA-based conclusions should be substantially tempered.

**Table 1** Minimum requirements for interpretable *Blastocystis* intervention studies (drug, probiotic, or combination)

| Domain | Item | Minimum requirement |
|---|---|---|
| **Isolate & diagnostics** | Parasite identity | **R**: Molecular confirmation of *Blastocystis* in inoculum/host (and assay details). |
| | Subtype/strain | **R**: Subtype identification (and allele/strain if feasible), or explicit justification for absence. |
| | Culture status | **R**: Xenic/mono-/axenic status; passage number; contamination checks. |
| **In vitro interventions** | Formulation control | **R**: Vehicle/excipient-only control for any commercial probiotic/sachet formulation. |
| | Culture chemistry | **R**: pH-matched control (and **SR**: redox/osmolarity matched where relevant). |
| | Live vs. non-live | **R**: Heat-killed (or equivalent non-viable) control to separate live activity from biomass effects. |
| | Mechanism separation | **SR**: Cell-free supernatant vs. washed cells to distinguish secreted metabolites from contact effects. |
| | Dose verification | **R**: Doses in absolute units (µg/mL; CFU/mL) and probiotic viability/CFU verification at start (and **SR**: end). |
| | Synergy claims | **R (if claiming synergy)**: Formal interaction assessment (e.g., checkerboard/FIC or equivalent), not descriptive comparisons. |
| **In vivo interventions** | Attribution controls | **R**: Uninfected + intervention arms (uninfected + drug; uninfected + probiotic) to separate clearance from immunomodulation. |
| | Study conduct | **R**: Randomisation and blinding stated for burden assessment, histology scoring, and immunostaining quantification (in animals). |
| | Burden quantification | **R**: Molecular burden measure (qPCR/ddPCR) alongside microscopy for clearance/near-clearance claims. |
| | Probiotic exposure | **SR**: Evidence of probiotic exposure/colonisation (stool CFU or strain-specific qPCR), or explicit statement if not assessed. |
| **Host readouts & pathology** | Immunostaining controls | **R**: No-primary, isotype, and known-positive tissue controls; defined quantification method. |
| | Pathology attribution | **R**: If structures in histology are presented as *Blastocystis*, organism-specific confirmation is required (ISH/immunolabeling or molecular correlation). **Morphology alone in routine sections is not sufficiently specific.** |
| **Reporting integrity** | Consistency check | **R**: Doses/units/group definitions consistent across abstract, methods, tables, and figures; resolve any mismatches prior to interpretation. |

R = required for parasite-specific claims; SR = strongly recommended;

### Overgeneralisation from a single, untyped human isolate

The study uses a single isolate from an IBS patient and does not perform molecular subtyping [4]. This limitation is acknowledged in the paper's limitations Sect [4]., but it is not reflected in the strength of the conclusions. Subtype and strain diversity are central to the biology and epidemiology of *Blastocystis* [5, 6], and drug responses vary across isolates in vitro [1]. A single, untyped isolate cannot support broad statements about "*Blastocystis* infection" or general therapeutic superiority.

### What should happen next?

This is not an argument against studying probiotics in *Blastocystis*. It is an argument for raising the minimum bar so that such studies are interpretable and reproducible. For this particular article, the immediate priority is to clarify and correct the inconsistent dosing and concentrations, and to resolve the IgA reporting contradictions transparently [4]. For the *Blastocystis* probiotic field more broadly, journals can rapidly improve signal-to-noise by requiring a short minimum controls checklist (Table 1) for intervention studies (in vitro and in vivo), alongside subtype reporting or a clear justification for its absence.

Table 1 was generated by synthesising recurring methodological issues highlighted here and in prior *Blastocystis* intervention work [1, 2], and by aligning the proposed minimum reporting items with widely adopted guidance for animal experiments (ARRIVE 2.0) and quantitative PCR (MIQE) [7, 8]. If journals in this field aim to be a home for work at the pathogen–microbiome interface, then enforcing these basics will yield dividends: fewer irreproducible claims, fewer over-interpreted findings, and a faster route to genuine mechanistic insight.

#### Abbreviations

| | |
|---|---|
| CFU | Colony-forming units |
| ELISA | Enzyme-linked immunosorbent assay |
| FITC | Fluorescein isothiocyanate |
| IBS | Irritable bowel syndrome |
| IgA | Immunoglobulin A |
| MTZ | Metronidazole |
| qPCR | quantitative polymerase chain reaction |

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ADT conceived and wrote the manuscript, EG and CRS reviewed and edited the manuscript.

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#### Data availability

Not applicable.

## Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

#### Authors' information

ADT and EG are the Chair and co-Chair of COST Action CA21105 [9] (*Blastocystis* under One Health), respectively, while CRS is the lead of working group 2.

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## Publisher's note