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A hypothesis-based hop microbiology laboratory module testing the plausibility of the mythical origin of the India Pale Ale

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ABSTRACT As one of the most famous fermented drinks in the world, beer is an especially relatable topic for microbiology courses. Here, we describe a short and easily adaptable module based on the antibacterial properties of hops used in brewing. By the 15th century, beer recipes included hops (the flower of the *Humulus lupulus* plant) as a bittering agent and antimicrobial. By the 19th century, the highly hopped Indian Pale Ale (IPA) became popular, and a modern myth has emerged that IPAs were invented to survive long ocean voyages such as from Britain to India. With that myth in mind, we designed a hypothesis-driven microbiology lab module that tests the plausibility of this brewing myth—namely that highly hopped beers possess enough antibacterial activity to prevent spoilage, while lowly hopped beers do not. The overall design of the module is to test the antimicrobial properties of hops using petri plates containing varying concentrations of hop extract. The module includes hypothesis generation and testing related to bacterial physiology and cell envelope morphology (hops are not equally effective against Gram-positive and Gram-negative bacteria) and to mechanisms of antimicrobial resistance (as beer spoilage bacteria have repeatedly evolved hop resistance). Pre- and post-assessment showed that students made significant gains in the learning objectives for the module, which encourages critical thinking and hypothesis testing by linking microbial physiology and antimicrobial resistance to an important and topical real-world application.

KEYWORDS hypothesis testing, laboratory exercise, bacterial cell envelope, antimicrobial resistance

Beer is rich in nutrients, yet spoilage is rare. In part, this is due to the antibacterial effects of ethanol, but bittering agents play a key role. Up until the Middle Ages, beer was bittered with gruit, a mixture of herbs mainly consisting of sweet gale and bog myrtle (1). While gruit had antibacterial properties (2), which is common for many spices and essential oils (3), by the 14th century, gruit was ultimately supplanted by hops, the flowers from the plant *Humulus lupulus*, as hops were cheaper and more consistent as a beer preservative (1, 4). In the 19th century, highly hopped beers such as the Indian Pale Ale (IPA) came into vogue, with a matter of historical dispute arising over their origin (5, 6). A common (though likely untrue) myth is that IPAs were popularized due to their ability to survive long ocean voyages (such as Britain to India, hence the name). With that myth in mind, we designed a hypothesis-driven module for a college microbiology lab course that tests the plausibility of the myth—namely that highly hopped beers possess enough antibacterial activity to prevent spoilage, while lowly hopped beers do not.

The overall design of the module is to test the antimicrobial properties of hops in a representative Gram-positive and Gram-negative bacterium on petri plates containing varying concentrations of hop extract. Hop iso-alpha acids are responsible for both the bitter flavor and the antibacterial activity of hops and are thought to act as ionophores that disrupt proton gradients (7, 8). Gram-negative bacteria are naturally hop resistant,

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likely due to the impermeability of the outer membrane to iso-alpha acids (9, 10). Thus, an added aspect of this module is the discussion of bacterial cell envelope morphology and generating hypotheses for whether hop antimicrobial activity would be more effective against Gram-positive or Gram-negative bacteria. Interestingly, despite the fact that Gram-negative bacteria are naturally resistant to hops, the vast majority of spoilage bacteria are Gram-positive bacteria (9). The evolution of hop resistance in Gram-positive bacteria is one major reason for this disparity (9, 10), and the final part of the module is a group discussion to generate plausible hypotheses for mechanisms of evolved hop resistance. Overall, this relatable module engages students in fundamental concepts in bacterial physiology, antibacterial activity, and the evolution of antibacterial resistance.

PROCEDURE

This two-part module was originally piloted as a standalone activity while waiting for beer to ferment for an upper-level lab course on the microbiology of brewing (11) and was refined to be adaptable to any college-level microbiology lab course. Learning objectives and assessments can be found in Appendices 2 and 3.

Safety issues

Biosafety level 1 category bacterial strains used were non-pathogenic *Escherichia coli* K12 strain MG1655 and a food-grade strain of *Lactobacillus buchneri*. During all lab activities, students should wear personal protective equipment (safety glasses, lab coats, and gloves). Concentrated iso-alpha acid extract is a moderate hazard (skin and eye irritant) and may cause allergic skin reactions.

Preparations before the module: media and bacterial culturing

A full supply list for the module is provided in Appendix 1. Iso-alpha acid (IsoHop) extract [30% (wt/wt)] can be purchased from Willamette Valley Hops (St. Paul, OR, USA). Beer bitterness is measured using the International Bittering Units (IBU) scale, with 1 IBU equaling 1 mg of iso-alpha-acids per liter of beer. Lysogeny Broth (LB) agar (for *E. coli*) and Lactobacilli MRS Broth (MRS) agar (for *L. buchneri*) are prepared using three different concentrations of iso-alpha acids: 10 IBU (lager level of hops), 50 IBU (IPA level of hops), and 100 IBU (double or imperial IPA level of hops), as well as a control plate lacking any hop extract (plain LB or MRS plates). We empirically determined via octanol extraction and UV-Vis spectroscopy (12, 13) that 1 IBU per L media corresponded to 26 µL of 30% (wt/wt) iso-alpha acid extract. A detailed protocol is provided in Appendix 7. Iso-alpha acid extract is added to the media prior to autoclaving (20 min at 120°C). Plates should be protected from light (iso-alpha acids are UV sensitive) and stored at 4°C for up to 1 week before use. Media recipes are provided in Appendix 4.

L. buchneri can be purchased from Wyeast Laboratories (Cat# 5335, Hood River, OR, USA), and *E. coli* K12 can be purchased from Carolina Biological Supply (Cat# 155068, Burlington, NC, USA). Bacteria are streaked from frozen stocks (20% glycerol) onto agar plates 1-week before the experiment. *E. coli* colonies arise after 1 day at 37°C and can be stored at 4°C until propagating overnight to saturation in liquid LB at 37°C with orbital shaking (270 rpm). *L. buchneri* colonies arise after 72 hours at 37°C and are propagated to the stationary phase (OD_{600nm} ~ 2) in liquid MRS at 30°C without shaking for 48 hours (14). On the day of the experiment, instructors prepare 1 mL working stocks for each student. For *E. coli,* saturated cells were diluted to an OD_{600nm} of 0.5 and then were further diluted 10^{-4} (~4×10^5 cells/mL). For *L. buchneri,* cells were diluted to an OD_{600nm} of 0.3.

Procedures during the module: part 1

The first part of the module consists of a short lecture on the history of hops, the mythical origins of IPAs, and bacterial cell wall physiology, which is then followed by a group discussion on hypotheses about whether Gram-positive or Gram-negative

bacteria are more likely to be affected by hops. A template for the lab lecture slides and additional resources for educators are provided in Appendix 5. Following the short lab lecture, students then prepare serial dilutions of bacteria to spot onto the plates with different levels of iso-alpha acids. Each student is provided with their 1-mL working stocks of bacteria, 1 mL each of sterile LB and MRS broth, and a sterile 96-well plate. Students prepare serial dilutions (10^{-4} dilution range) in the 96-well plate by transferring and mixing 10 µL of culture with 90 µL of sterile media, followed by spotting 4 µL (*L. buchneri*) or 20 µL (*E. coli*) of the whole dilution series onto the appropriate plates (one control plate and three hop extract plates). *E. coli* plates are incubated at 37°C for 24 hours, while *L. buchneri* plates are incubated at 37°C for 72 hours. Once colonies have arisen on the plain-media control plates, the plates can be stored at 4°C until the second part of the module.

Procedures during the module: part 2

The second part of the module begins with students interpreting their results in light of their original hypotheses. Figure 1 depicts representative results, with the Gram-negative *E. coli* being resistant to iso-alpha acids and Gram-positive *L. buchneri* showing growth only at low (10 IBU) levels of iso-alpha acids. These results illustrate that hops specifically inhibit Gram-positive bacteria, while also providing some credence to the notion that IPA levels of hops could reduce beer spoilage during long ocean voyages. Finally, it is pointed out that Gram-positive bacteria can evolve hop resistance, which is still a problem for industrial brewers, and students discuss as a group plausible molecular mechanisms for how hop resistance may arise. Possible future modifications are included in Appendix 8 and include a semi-quantitative colony scoring system that allows for statistical analyses, as well as how generative artificial intelligence can be used as a learning tool. We provide example prompts and answers using ChatGPT3.5 for possible questions students may ask related to the module while noting when the provided answers contradict published sources.

CONCLUSION

We created a microbiology lab module that can be easily adapted and integrated into a college-level microbiology or molecular biology lab course. This module effectively encourages critical thinking and hypothesis testing by linking microbial physiology and antimicrobial resistance to an important and topical real-world application.

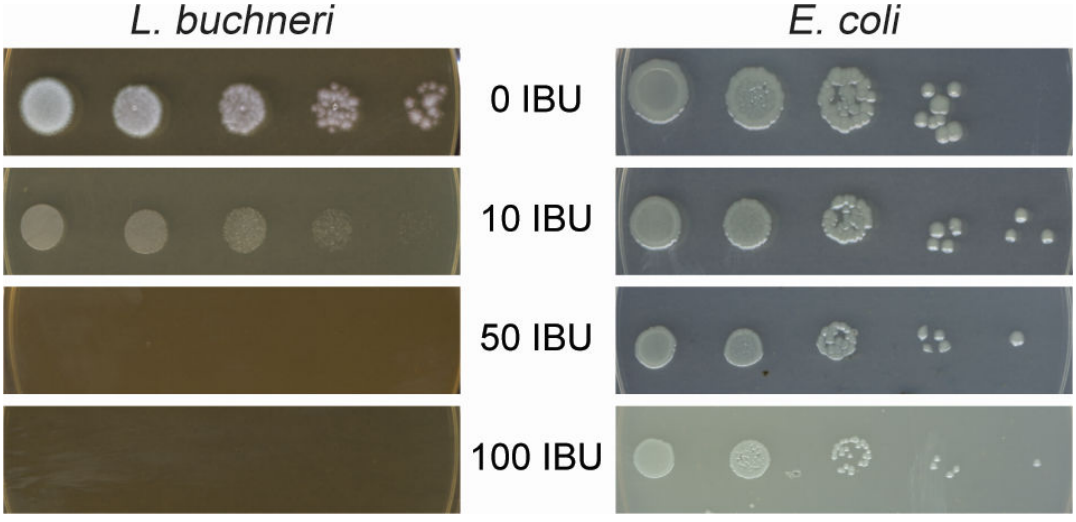

FIG 1 Representative results of serial dilutions of *L. buchneri* and *E. coli* on plates containing the indicated levels of iso-alpha acids.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (jmbe00020-24-s0001.docx). Supplemental appendices 1 through 8

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