Summary of 2021 IAFP Roundtable Discussion on "Opportunities and Challenges: Developments in *Clostridium botulinum* Challenge Studies"

Kristin Schill (Associate Scientist for the Food Research Institute, upper right) moderated an IAFP roundtable discussion on "Opportunities and Challenges: Developments in *Clostridium botulinum* Challenge Studies" at the 2021 IAFP Annual Meeting. The discussion featured international experts from industry, academia, and government discussing the unique challenges that face food companies who need to conduct *C. botulinum* challenge studies and highlighted progress in the development of new surrogates for the organism.

As highlighted by Schill, the diversity and toxicity of the organism complicate *C. botulinum* challenge studies. The organism's diversity means it is extremely unlikely that a single universal surrogate could be used for all *C. botulinum* challenge studies. The organism's toxicity means that governments worldwide consider *C. botulinum* a select agent and have established strict requirements for those working with the pathogen, making testing difficult and expensive.

The lack of correlation between *C. botulinum* growth and toxin production means that testing in challenge studies requires testing for the toxin, not simply bacterial growth. Another problem: In the U.S., the FDA maintains that the gold standard assay for botulinum toxin detection in foods is the mouse bioassay, further limiting the labs that can conduct *C. botulinum* challenge studies to those with access to approved protocols and facilities. Indeed, speaker Michael Peck (QIB Extra) estimated only 10 laboratories in the world (which includes the Food Research Institute) are capable of conducting *C. botulinum* food challenge studies. This lack of laboratories working on *C. botulinum* creates an additional challenge: progress in developing methods to genetically manipulate the organism has been slow.

As discussed by Maxine Roman (Kraft-Heinz), consumer demand for fresher, less processed foods with fewer preservatives and longer shelf-lives means that food companies have

increasing numbers of new or modified products formulations. Chilled foods have also become very popular, according to Stephen Grove (Nestle), and such foods carry the potential for non-proteolytic *C. botulinum* growth and toxin production. Food manufacturers want to know how such challenge studies can be conducted less expensively yet still appropriately.

The use of surrogate strains in place of toxigenic *C. botulinum* can reduce costs during initial screenings of new formulations. Surrogates are needed for two very different types of *C. botulinum*: the proteolytic (Group I) strains, which grow optimally at 37 to 42°C, and the nonproteolytic (Group II) strains, which grow optimally at 25 to 30°C but can grow at temperatures as low as 2.5°C. As outlined by Peck, there are three categories of *C. botulinum* surrogates.

- "Genuine" natural strains that lack the toxin gene (such as PA3679, used as a surrogate for proteolytic strains) represent a tried-and-tested approach for thermal processing validation studies. However, because such strains lack toxin, only the organism's growth but not development of toxicity can be measured in growth inhibition studies in foods stored at slight temperature abuse. If such strains grow to high levels in a food product formulation during testing, it might be reasonable to assume that toxin is present and to abandon that formulation. However, lack of growth isn't enough to demonstrate a product would not allow toxin formation, since it is not clear if toxin can be produced by *C. botulinum* under conditions of minimal growth. Furthermore, enumeration of nonproteolytic *Clostridium botulinum* by plating on solid agar is unreliable, and mesophilic PA3679 cannot be used to predict inhibition of psychrotrophic strains.
- Another approach is to use *C. botulinum* strains in which the toxin gene has been intentionally inactivated or deleted. Sabine Pellett (University of Wisconsin-Madison) discussed work that her laboratory has done in developing "ClosTron" mutants. These mutants have the toxin gene deleted and can be selected for by the incorporation of antibiotic resistance markers. However, such strains are still considered select agents at this time. More advanced CRISPR Cas9 methods for *C. botulinum* are still being developed and will greatly facilitate genetic manipulation of the organism.
- Different bacterial species (for example, *Listeria monocytogenes* or *Bacillus cereus*) with similar growth characteristics could potentially be used as indicators of formulation stability under some circumstances.

As discussed by the group, the ideal surrogate would be a strain that lacks toxicity but still expresses a detectable reporter gene, with some assurance that reversion of the strain to one expressing the native toxin cannot occur. The best reporter gene could be a version of the toxin itself, attenuated to make it non-toxic. The Group II (nonproteolytic, psychrotrophic) *Clostridium* Beluga E1 strains, which fit these criteria, have attracted interest. But questions remain as to how atoxic such stains would have to be for their acceptance as surrogates and not be considered select agents.

Jenny Scott (FDA) commented that while FDA has no regulations specifically addressing surrogates, the preventive controls for human foods rule requires validation of process controls, which could include challenge studies for *C. botulinum* that potentially utilize

surrogates. The use of surrogates for *C. botulinum* is complicated because it is really a group of organisms with vastly different characteristics. A surrogate may be useful in mimicking *C. botulinum* in certain characteristics (heat resistance, for example), but different surrogates may be necessary for other characteristics related to growth inhibition in foods. Bottom line: FDA will look at the characteristics of the surrogate relevant to the food if *C. botulinum* is the hazard of concern and a surrogate is used in validation data for a food product.

The detection of toxin by the mouse bioassay is a significant obstacle when conducting *C. botulinum* challenge studies, especially given the increased demand for transparency and lack of tolerance for animal studies by consumers. In Europe, ELISA assays are used instead to detect *C. botulinum* toxin in foods, and these assays have demonstrated equivalent or better sensitivity than the mouse bioassay. However, commercial availability of quality antibodies to conduct such testing is limited. Development of a mass-spectrometry endopeptidase assay for the toxin is also underway. These newer methods can potentially replace the mouse bioassay in the U.S. in coming years, but not until they are validated in a variety of food products.

The roundtable experts discussed strategic approaches to consider when testing for the potential development of *C. botulinum* toxicity in new product formulations. Grove, Pellet, and Roman suggested predictive models, while are mostly based on growth and not toxin formation, can be a useful starting place to narrow down possible formulations. Surrogates, particularly cocktails of atoxic strains, can also be used to screen for the most promising formulations for further validation testing. Movement to replace the mouse bioassay for toxin is a regulatory goal (per Scott) and will improve consumer acceptance of *C. botulinum* challenge studies, but the number of laboratories with capabilities to handle the agent will still be limited.