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Is there a causal relationship between trehalose consumption and *Clostridioides difficile* infection?

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Key words

Clostridioides difficile, trehalose, mutant variant trehalase, CDI

Abstract

Purpose of review

Trehalose metabolism appears to play a role in the pathogenicity of some microbes. It has been claimed that trehalose consumption may be a risk factor for *Clostridioides difficile* infection (CDI), but the evidence for a causal link is contentious.

Recent findings

Epidemic ribotypes of *C. difficile* harbour mutations or have acquired extra genes that mean these strains can utilise lower concentrations of bioavailable trehalose, providing a competitive metabolic advantage in some CDI animal models. By contrast, evidence has emerged to show that trehalose-induced microbiota changes can help protect/reduce CDI in other models. Additionally, *C. difficile* trehalose metabolic variants are widespread amongst epidemic and non-epidemic ribotypes alike, and the occurrence of these trehalose variants was not associated with increase disease severity or mortality.

Summary

Currently, there is no proven causal association between the incidence or severity of human CDI and the presence of trehalose metabolism variants. Furthermore, microbial metabolism reduces trehalose bioavailability, potentially removing this competitive advantage for *C. difficile* trehalose metabolism variants. Taken together, trehalose consumed as part of a normal diet has no increased risk of CDI.

Key Points

- Human consumption of trehalose has increased since the year 2000 following an increased use of this disaccharide in the food industry.
- Consumed trehalose is metabolised by host epithelial, and microbial produced, trehalases.
- Genetic variants of *C. difficile* confer enhanced utilisation of low concentrations of bioavailable trehalose.
- Trehalose metabolism variants are widespread amongst clinical *C. difficile* isolates.
- Clinical investigations found no association between *C. difficile* trehalose metabolism variants and *C. difficile* infection severity.

Introduction

Trehalose is a disaccharide that consists of two glucose subunits linked via an α, α-1, 1-glycosidic bond, with many commercial, industrial, and biomedical uses. This disaccharide is found in a wide variety of organisms, including plants, invertebrates, insects, fungi/yeast, and bacteria. The widespread utilisation of trehalose in nature can be attributed to its interesting biochemical properties of trehalose, which include high hydrophilicity, resistance to acid hydrolysis, and chemical stability. In yeast and fungi, trehalose is found at high concentrations in spores and fruiting bodies, and rapidly disappears upon germination, suggesting this stored trehalose acts as a carbon or energy source. Whereas in plants, trehalose has a multifaceted role: it is involved in starch synthesis, the response of plants to abiotic stress, and as a signal metabolite in plant interactions with micro-organisms (as reviewed in [1].

Some foods naturally have low levels of trehalose, such as mushrooms, honey, bread, and beer. However, trehalose exhibits some physical properties that make it an important food additive. Trehalose has a relative sweetness of 45 % to that of sucrose, this disaccharide does not show Millard (browning) reaction with amino compounds, and it has a high thermostability and wide pH stability range [2]. These properties make trehalose ideal for many applications within the food industry because it maintains food quality. However, the use of trehalose as a food additive was not viable until a more efficient process of manufacture was developed. Hayashibara Company Ltd discovered that enzymatic degradation of corn starch produces a high yield of trehalose and represented an inexpensive methodology for trehalose production [3].

As a food additive, trehalose is used in many baked goods (biscuits, cakes, confectionary etc), ice-cream, instant pasta/rice/noodles, and processed fruit (jams and fillings) [4]. Those same biochemical properties that make trehalose useful in the food industry also make trehalose a good supplement used in the medical/cosmetic industry. Trehalose ability to enhance the stability of proteins in solution has led to its use in monoclonal antibody products such as those produced by Genentech [4]. Similarly, crystalline trehalose does not readily absorb water and is used as a coating on tablets to reduce stickiness and enhance stability. The use of trehalose is also gaining attention for use in reducing the effects of metabolic diseases, such as atherosclerosis [5*], and post infarct cardiomyopathy [6]. Trehalose was found to reduce the aggregation of mutant Huntingtin protein, preventing the onset of Huntington's disease [7]. Within the cosmetic industry, trehalose is a component of skin moisturisers, shampoos, and deodorants.

The development of financially viable manufacturing processes approximately 15-20 years ago, and the incorporation of trehalose into different applications, potentially exposes some humans to an increased level of this disaccharide.

Microbial metabolism of trehalose

The accumulation of trehalose in microbial cells has functional roles in different microbial genera. In *Mycobacterium* and *Corynebacterium* species, trehalose acts as a structural component in the cell wall, through incorporation into glycolipids, such as trehalose-dimycolate [8]. Trehalose has been proposed to act as general stress protectant in yeast/fungi, including *Candida* species, especially in response to dehydration, osmotic stress, and thermal stress [9, 10]. However, the most common use of trehalose is as a carbon/energy source during metabolism. Microbes can metabolise trehalose through 5 different pathways, as shown in **Figure 1**. Depending on the genetic determinants within a microbial genome, trehalose can be synthesised from maltose, maltooligosaccharides, and phosphorylated glucose/nucleotide conjugated glucose. Each of these biosynthetic pathways are equally distributed amongst Eubacteria, except for TreH pathway, which is most commonly found in Archaea [11]. Within spores of fungal/yeast and bacterial species (*Streptomyces* spp.), trehalose acts as an early use energy source during germination of these spores; trehalose can account up to 10 % dry weight of *Neurospora tetrasperma* spore cells [12]. Disruption of the trehalose biosynthetic pathways in *Saccharomyces cerevisiae* leads to increased susceptibility to heat shock, inability to grow at temperatures above 34 °C, and an inability to grow on glucose and other fermentable sugars [10].

Microbial metabolism of trehalose, and derivatives, occurs via trehalases, to produce glucose, which can be used during glycolysis. Species of *Bacillus* use trehalose as a source of carbon and energy during exponential growth; similarly, *Escherichia coli* uses trehalose as a carbon source in an osmolarity-dependent fashion [13]. The rise in next generation sequencing technologies has seen an explosion of microbial genomes annotated, which, in turn, has expanded our knowledge of the distribution of trehalase genes. The UniProt protein sequence repository [14] has thousands of trehalase protein sequences from a wide range of microbial species, including those bacterial species commonly found as part of the human intestinal microbiota and human pathogens. Indeed, trehalose metabolism appears to play a role in microbial pathogenicity. Mutational disruption of the trehalose biosynthetic pathway in *Mycobacterium tuberculosis* lead to severe growth defects and decreased *in vivo* virulence [15]. Similarly, biofilm formation and capsule production in *Klebsiella pneumoniae*, and mouse gastrointestinal colonisation are all impaired if the trehalose catabolism pathway (*treBC*) is disrupted [16]. Additionally, it should be noted that mammals do not possess any trehalose biosynthetic pathway; however, host-produced trehalases can be found within the brush boarder of the small intestine, which reduces the amount of bioavailable trehalose entering the large intestine.

These data show that trehalose is associated with several microbial biological processes including, metabolism, sporulation, germination, and virulence. In pathogenic microorganisms, the ability to synthesise and/or metabolise trehalose is important for full

virulence; however, as trehalose is associated with multiple biological processes, one must delineate the importance of this compound towards cell survival over pathogenesis.

Ability of *Clostridioides difficile* to metabolise trehalose

One such pathogen capable of metabolising trehalose is *C. difficile*, which is a spore-forming, anaerobic Gram-positive bacillus that is the leading infective cause of antibiotic-associated diarrhoea. Ingested *C. difficile* spores are highly resistant to the gastric pH of the digestive system, but stay quiescent due to the resident microbiota. However, if the microbiota is disrupted, i.e. through the action of antibiotics, *C. difficile* spores germinate, outgrow, and cause disease, *C. difficile* infection (CDI). *C. difficile* isolates can harbour up to 3 toxins, TcdA, TcdB and CDT toxin [17], which are responsible for the destruction of the epithelial layer and symptom manifestation, possibly including pseudomembranous colitis, toxic megacolon, and death. Since 2000, worldwide CDI infections increased, notably following the global spread of an epidemic strain, ribotype (RT) 027/BI/NAP1, which caused increased levels of patient morbidity and mortality. The widespread transmission of this ribotype has been attributed to the acquisition of antibiotic resistance, namely fluoroquinolone resistance [18]. Expansion of other epidemic *C. difficile* ribotypes, such as RT017 and RT078, is a continuing global trend [19]. However, it is unclear what genetic factors are involved in the pathogenicity and transmission of these epidemic ribotypes over other circulating ribotypes. Against this background, could increased trehalose utilisation provide an answer?

All strains of *C. difficile* harbour trehalase (TreA), a phosphotrehalase enzyme capable of catabolising trehalose, which is under the control of an upstream transcriptional regulator (TreR) [20]. TreA catabolises trehalose-6-phosphate into glucose and glucose-6-phosphate and is essential for *C. difficile* growth on trehalose-containing media [21**]. However, some *C. difficile* ribotypes, such as RT027, grow more effectively in the presence of low trehalose concentations (50 µM), compared with some other ribotypes. Sequence analysis of the genetic loci encoding trehalase showed a single nucleotide polymorphism (SNP) within *treR* that allows derepression of *treA*, in RT027 isolates that is responsible for the enhanced growth on low concentrations of trehalose. Ribotypes with this mutation have a selective *in vivo* advantage in the presence of trehalose, and increased virulence [21**]. The increased virulence was attributed to increased production of TcdB; however, caution is needed when extrapolating these results.

C. difficile toxin expression is tightly controlled through many different elements, one of which is catabolite control protein CcpA [22]. This pleotropic regulator can coordinate the genes involved in metabolism and virulence and represses toxin expression in the presence of excess glucose levels [23]. Thus, *treA* null mutants, potentially, could have a CcpA-mediated indirect effect on toxin levels. A separate SNP mutation in *treR* of RT017 isolates has been seen, which also enabled growth in the presence of low trehalose levels [24]. In RT078, enhanced trehalose utilisation is conferred through a different mechanism. In this ribotype, a four gene cluster has been horizontally acquired consisting of a second trehalase

(TreA2) and associated repressor gene (TreR2), a putative glycan debranching enzyme (TreX), and a potential trehalose-specific PTS system IIBC component transporter (PtsT) [21**]. Mutational analysis of the transporter gene showed that this gene conferred enhanced growth on low levels of trehalose. Another line of evidence for enhanced trehalose utilisation by these *C. difficile* ribotypes has been shown when using degradation-resistant analogues of trehalose, lactotrehalose and mannotrehalose, which reduce the ability of trehalose-cluster ribotypes to metabolise trehalose [25].

The increased usage of trehalose in the early part of the century, and the increased competitive nature that trehalose mutations confer to *C. difficile* epidemic strains, led to a hypothesis that this disaccharide has contributed towards the emergence of these epidemic ribotypes [26, 27]. However, are trehalose mutations associated with enhanced pathogenesis in a clinical setting?

Trehalase mutations alone are not sufficient for enhanced CDI

In a recent study, we wanted to explore the effect of trehalose on the human microbiota and the consequential induction of CDI using a previously validated *in vitro* human gut model [28]. We found that the human microbiota quickly remodelled to metabolise all bioavailable trehalose, and antibiotic-induced changes to the microbiota, along with continued trehalose instillation, did not result in induction of CDI compared with glucose or saline instillation [29**, 30**]. In parallel, Zhang *et al*., [31*] used a mouse model of severe CDI, where they investigated the effects of trehalose and lactotrehalose on the microbiome and CDI severity. Here, the authors describe that orally dosed trehalose actually reduced *C. difficile* abundance, and this was associated with lower levels of detectable TcdA. These studies suggest that microbial metabolism plays an important role in the bioavailability of trehalose, even during antibiotic exposure. This is unsurprising given the abundance of trehalases encoded by the intestinal microbiota and how the microbiota remodel to utilise this carbon source.

In an effort to understand the distribution of trehalose mutations amongst clinical cases of CDI and the association of these mutations with disease severity, Eyre *et al* [29**] analysed over 5000 clinical *C. difficile* genomes. The *treR* point mutants described are more widespread than previously thought, distributed amongst *C. difficile* isolates from clade 2 and 4, whilst the four-gene cluster was variably present in genomes from all 5 clades [29**, 32*]. Interestingly, amongst the *C. difficile* RT015 clinical isolates (n=208) the four-gene cluster was variably contained; however, there was no association between the presence of this gene cluster and enhanced mortality (within 30 days) for this ribotype, when considering all clinical parameters. Similarly, Saund *et al*., [32*] analysed the clinical data from 1144 CDI patients and the genomes of the corresponding isolated *C. difficile* isolates. The authors concluded that there was a lack of association between trehalose utilisation variants and severe CDI outcome in hospitalised patients. In both studies, trehalose variants were much more widespread than previously thought, suggesting the ability to utilise low

concentrations of trehalose was acquired prior to the recent increase in trehalose usage. A limitation of these studies is that the isolates analysed were predominantly from Europe or the US; it remains to be seen if there is an association between trehalose utilisation variants from Asia, where trehalose usage is higher, and increased CDI severity.

Notably, as highlighted herein, these data show a disparity between mammalian models of infection and the heterogeneity (both genetically and on a microbiome scale) of the human population, and the need to use a variety of models that can capture/simulate human disease [33].

Conclusion

Increased usage of trehalose at the turn of the century has been proposed to have contributed to an increase in the number and severity of *C. difficile* epidemics caused by strains that can better utilise low concentrations of trehalose. However, this hypothesis was not supported by clinical data from CDI patients, or when using a clinically reflective human gut model. It should be noted also that the incidence of certain epidemic strains that can utilise low levels of trehalose has decreased, despite the continued usage of trehalose in the food/cosmetic/pharmaceutic industries. It appears unlikely, therefore, that the success of epidemic *C. difficile* ribotypes (RT027, RT078 and RT017) has been driven by a change in their capacity to utilise trehalose.

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None

Conflicts of interest

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

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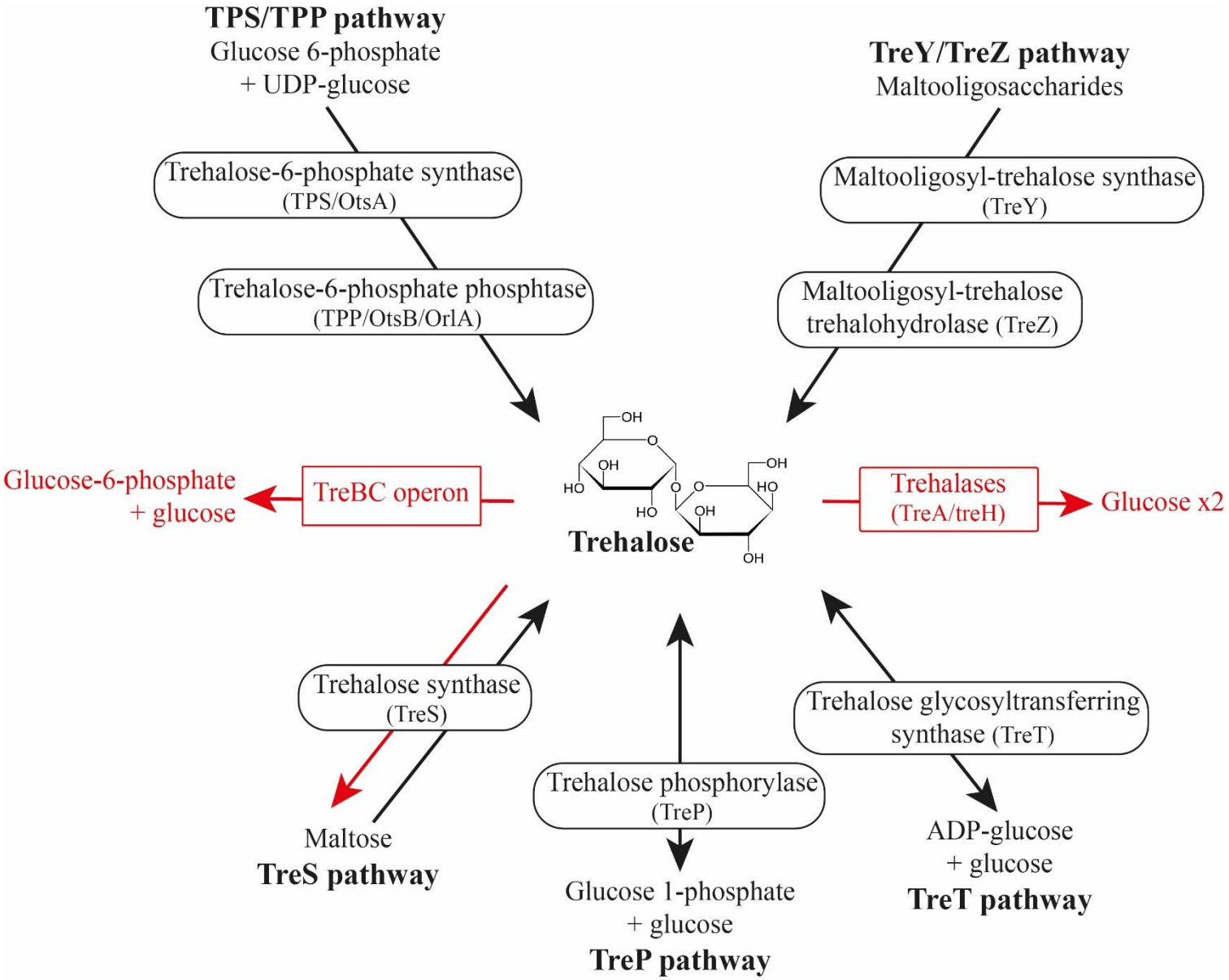
Figure legend

Figure 1. Bacterial trehalose metabolic pathways. Trehalose biosynthesis pathways are shown with black arrows, and trehalose catabolic pathways are shown with red arrows. Enzyme names are shown in the parenthesis. Adapted from [34].


TPS/TPP pathway
Glucose 6-phosphate
+ UDP-glucose
Trehalose-6-phosphate synthase
(TPS/OtsA)
Trehalose-6-phosphate phosphtase
(TPP/OtsB/OrlA)
TreY/TreZ pathway
Maltooligosaccharides
Maltooligosyl-trehalose synthase
(TreY)
Maltooligosyl-trehalose
trehalohydrolase (TreZ)
Glucose-6-phosphate
+ glucose
TreBC operon
Trehalose
Trehalases
(TreA/treH)
Glucose x2
Trehalose synthase
(TreS)
Maltose
TreS pathway
Trehalose phosphorylase
(TreP)
Glucose 1-phosphate
+ glucose
TreP pathway
Trehalose glycosyltransferring
synthase (TreT)
ADP-glucose
+ glucose
TreT pathway