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RECEIVED 13 April 2024

ACCEPTED 12 July 2024

PUBLISHED 30 July 2024

CITATION

Marian C, Sanjar F, Maxwell C, Sabitu F,
Hubbard N, Ratib N, Vassilev N and
Sansinenea E (2024) A paradigm for the
contextual safety assessment of agricultural
microbes: a closer look at *Klebsiella variicola*.
Front. Ind. Microbiol. 2:1412302.
doi: 10.3389/finmi.2024.1412302

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A paradigm for the contextual safety assessment of agricultural microbes: a closer look at *Klebsiella variicola*

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Adopting a risk assessment paradigm that is contextualized and strain-specific will be critical to enabling the continued development and safe use of microbes, particularly bacteria, in numerous industries and applications. An overly simplistic approach of labeling bacterial species as either harmful or beneficial is ill-suited for the complexities of their interactions with hosts and other microbes, where the lines between friend, foe, and innocent bystander are often unclear. Many such nuanced relationships have been described in human microbiome studies, illustrating the inherent challenges of defining bacterial safety. Any effective risk assessment framework must take into account bacterial niche and environment, fitness, host health, route and extent of exposure, and strain characterization. *Klebsiella variicola*, a diazotrophic soil bacterium isolated around the world, has been the subject of increasing interest on both environmental and clinical fronts, and has been used commercially as a biofertilizer on millions of farm acres. Here we review its population structure, relevance in clinical and environmental settings, and use as a biofertilizer in light of the risk assessment framework described.

KEYWORDS

safety assessment, commensal microbiota, biotechnology, microbiome dynamics, diazotroph, biofertilizer, sustainable agriculture, *Klebsiella variicola*

Introduction

As tiny living factories, bacteria have provided a host of applications in industries ranging from food to fuels (Akoijam et al., 2022). They hold the potential to make tremendous contributions toward a more sustainable future (Robbins, 2021), but evaluating their safe use remains a challenge. One of the pitfalls of assessing bacterial safety is creating a false dichotomy wherein species are considered categorically harmful or

beneficial. In reality, the relationship between bacteria and their hosts is incredibly complex and dynamic, and the concept of bacterial safety must be viewed along a spectrum that is highly niche-dependent, strain-specific, and never static. The need for this conceptual shift in safety assessment has been illustrated and informed largely by human microbiome research, and its relevance to other domains such as industry and agriculture is deeply evident (Sansinenea, 2022).

Reframing the concept of bacterial risk

Bacteria are a pervasive presence in the human body. The average adult carries roughly 1 kilogram of bacteria in their gut, many of which are uncultivable, and it is estimated that less than 1% of all microbes are considered human pathogens (Microbiology by numbers, 2011). Many bacteria are beneficial – even vital – to our systemic health, yet the pathogenic few have devastated human populations throughout history, and millions still die every year of infectious diseases, despite tremendous strides made with the use of antibiotics. Given our confounding relationship with bacteria, the desire to identify and eliminate bad actors is not surprising. However, it is becoming increasingly apparent that many members of the human microbiota cannot easily be classified as friend or foe, and attempts to label them as such are often shortsighted (Jochum and Stecher, 2020). Human microbiomes bring to light multifaceted, often surprising associations, wherein the same bacterial species can be considered commensal, mutualistic, or pathogenic, depending on its niche access, environmental conditions and resources, and specific strain genotype.

For example, *Helicobacter pylori* has colonized the human stomach for millennia, has been considered a gastric pathogen for the last few decades, and more recently appears to be protective against certain diseases, including childhood-onset asthma (Chen and Blaser, 2008; Blaser, 2010). Attempting to reconcile these observations raises numerous questions. Is *H. pylori* a driver in development or progression of gastric disease, or simply a passenger? Is pathogenicity only linked to certain strains or genotypes of this species? Might eliminating it from its long-held niche have unintended consequences? Additional research is needed to answer these questions, but what is clear is that humankind's relationship with this microbe is more complex than previously thought.

Enterococcus faecalis is a ubiquitous human and animal gut microbe that can behave as a commensal, probiotic, or pathogen, depending on the strains and environment (Franz et al., 2011; Hanchi et al., 2018; Braïek and Smaoui, 2019; Krawczyk et al., 2021). It is widely used as a starter culture for fermented dairy products, can have protective effects against foodborne pathogens, and has been evaluated as an adjuvant therapy for sinusitis in children (Kitz et al., 2012; Hanchi et al., 2018). However, outside of its normal food and gut-associated niches, it is a frequent cause of hospital-acquired bacteremia, urinary tract infections, and infectious endocarditis,

posing an increased risk to immunocompromised patients with comorbidities, catheters, and history of broad-spectrum antibiotic use (Arias and Murray, 2012; Dubin and Pamer, 2017). Several studies have been carried out to determine the differences between clinical and food-derived isolates, especially with respect to genes associated with virulence and antibiotic resistance (Eaton and Gasson, 2001; Franz et al., 2001; Semedo et al., 2003). While such genes were more frequently found in clinical isolates, they were also present in the genomes of nonpathogenic food isolates, and some (e.g., *gelE*) were phenotypically silent under similar conditions. In short, the species defies a clear-cut risk designation, and the safety of *E. faecalis* must be evaluated by individual strain.

Escherichia coli is yet another species in which diverse roles are observed due to strain-to-strain genetic variation. Certain *E. coli* serotypes introduced during vaginal delivery typically colonize a newborn's gastrointestinal tract within hours of birth, are recognized as critical symbionts for healthy microbiome development, and persist throughout life (Blount, 2015). One particular strain, marketed under the name Symbioflor[®] 2, has been approved as a treatment for irritable bowel syndrome (IBS) in the European Union (EMA recommends that Symbioflor 2 can continue to be used for irritable bowel syndrome, 2017). Other serotypes, such as the enterohemorrhagic colitis causing O157:H7, are highly pathogenic, possessing specific combinations of virulence-related factors that render them capable of causing potentially fatal kidney damage (Kaper et al., 2004). Once again, the line between friend and foe is nuanced, and attempting to apply a single risk designation for the entire *E. coli* species is futile.

Other human gut bacteria are widely viewed as categorically safe, and have long been used as probiotics and food additives. The global probiotic market is valued at over \$50 billion USD, and more than 1,000 clinical trials have been conducted to study the benefits of probiotics in over 700 conditions and diseases (Dronkers et al., 2020; Probiotics Market Size, 2021). *Lactobacillus* and *Bifidobacterium* are the most commonly used genera in probiotics and fermented foods, with well-documented health benefits (Probiotics - Health Professional Fact Sheet, 2023). Lactobacilli dominate healthy vaginal microbiota, and their depletion is associated with several disease states, including infection, pelvic inflammatory disease, and infertility (Ravel et al., 2011; Kroon et al., 2018; Younes et al., 2018). Bifidobacteria species are among the most dominant gut colonizers of breastfed infants, and their presence has a profound positive impact on health throughout life, with inverse associations observed for obesity, metabolic disease, allergy, and infection (Bäckhed et al., 2015; Arboleya et al., 2016; Turroni et al., 2020; Stuijvenberg et al., 2022). Yet even *Lactobacillus* and *Bifidobacterium* species can become pathogenic when introduced outside of their normal niches, especially in individuals with comorbidities or risk factors such as age (specifically neonates and the elderly), and history of broad-spectrum antibiotic use (Pathak et al., 2014; Butta et al., 2017; Esaiassen et al., 2017; Rossi et al., 2019).

When considering these and a myriad of similar examples from plant, animal and soil microbiomes, the picture of bacterial risk that begins to emerge is one with a great deal of nuance. From it, we can make several observations:

1) The importance of bacterial niche specificity and environmental stimuli cannot be overstated. What causes disease in one context may provide an essential benefit in another. Symbiotic bacteria are highly niche-specific, living in delicately balanced communities that have co-evolved with their hosts. Perturbations to this balance – known as “dysbiosis” – can have deleterious effects, and the potential risks of indiscriminately eradicating a species that evolved to inhabit a specific niche are clear. In such communities, fine-tuning, rather than wholesale elimination of a species, should be the prevailing approach.

2) In a sense, all microbes are opportunists. A host experiencing disease, dysbiosis, or weakened immune function provides an environment where even its closest bacterial allies can become enemies. In such an environment, it is also possible for passengers in a disease to be mistaken for drivers. Considering overall host health and unraveling specific roles in disease are difficult but necessary steps.

3) Different strains of the same species can vary widely in their genotypic and phenotypic profiles, and as a result, may have remarkably different behaviors. Therefore, risks must by necessity be evaluated at the individual strain level.

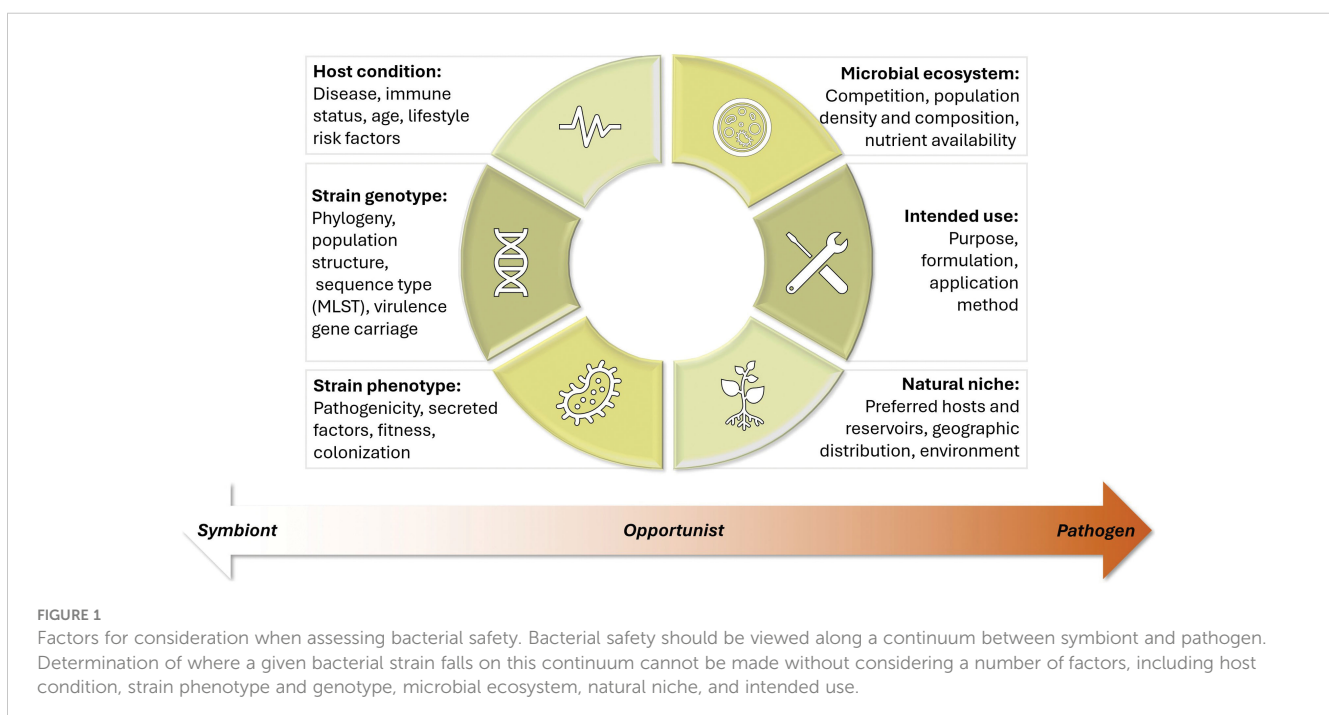
4) Given the constant evolution of bacteria in response to environmental cues and pressures, ongoing surveillance is necessary.

Clearly, bacterial behavior and impact in any given habitat are the results of a delicate balance of environmental, host health, and strain-specific factors (Figure 1). In such a complex, ever-shifting landscape, defining which bacteria are considered safe and under which circumstances requires moving beyond simplistic labels of “good” or “bad.” Accurately evaluating bacterial risk requires a wide-angle lens, and context is critical. This paradigm shift is gaining increasing recognition (Wiles and Guillemin, 2019; Jochum and Stecher, 2020), and its widespread adoption will create a path forward for bringing countless new microbial products and technologies to life.

Klebsiella variicola

An illustration of this contextualized approach to safety evaluation in the agricultural arena can be drawn from the diazotrophic bacterium *Klebsiella variicola*, a member of the *Klebsiella pneumoniae* species complex (KpSC) which has been successfully developed for use as a biofertilizer. First isolated from healthy banana plants and identified as a novel nitrogen-fixing species in 2004, *K. variicola* has gained increasing attention in recent years as a bacterial species of both clinical and environmental interest (Rosenblueth, 2004; Rodríguez-Medina et al., 2019; Duran-Bedolla et al., 2021). Like other members of the *Klebsiella* genus, *K. variicola* is a gram-negative, facultative anaerobic non-spore-forming bacterium, with plants considered to be its natural reservoir (Rosenblueth, 2004). It has widespread geographic distribution and can colonize a wide range of diverse niches. Strains of *K. variicola* have been isolated from many sources, including leaf-cutter ant fungus gardens (Pinto-Tomas, 2009), fecal samples of healthy breastfed infants (Rosales-Bravo et al., 2017; Gómez et al., 2021), the traditional fermented Mayan beverage pozol (Rizo et al., 2020), and industrial effluents (Afzal et al., 2017). Gut carriage of *K. variicola* and other KpSC member species has also been confirmed in healthy adults (Raffelsberger et al., 2021). Furthermore, in-house sequence-based screening of the publicly available Human Microbiome Project database (Human Microbiome Project Data Portal, 2013) for the presence of KpSC members in healthy human subjects revealed frequent detection of these microbes across different body sites, particularly oral (Figure 2).

The ability to inhabit these different niches stems from the species’ remarkably high genetic diversity. Molecular genotyping of *K. variicola* has identified over 150 sequence types to date, with some strictly isolated from one niche (plant, animal, or human),



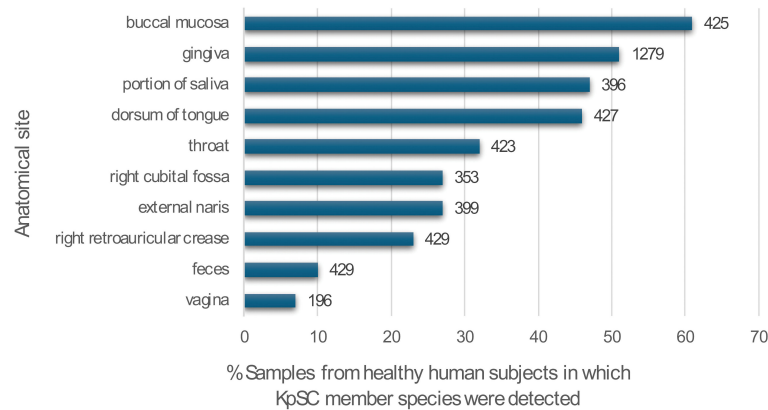


FIGURE 2

Prevalence of KpSC member species detected across different anatomical sites in 5,906 sample sequences collected from healthy adults and deposited in the Human Microbiome Project database (Human Microbiome Project Data Portal, 2013). Bars labeled with sample size from each site. Screening methods can be found in Supplementary File 1. Sample metadata can be accessed in Supplementary File 2.

and others identified across kingdoms (Guo et al., 2018; Barrios-Camacho et al., 2019; Morgado et al., 2022). Based on their biosafety profiles, *K. variicola* strains have been identified with containment level assignments of BSL1 and BSL2 – for example, strains ATCC F2R9 and LMG 23571, respectively (ATCC F2R9, 2001; LMG 23571, 2004).

Genomic differences can significantly alter the behavior and niche of different *K. variicola* strains, but attempting to make associations quickly becomes problematic. For example, a phylogenetic core genome study of 145 primarily clinical *K. variicola* isolates showed two distinct lineages, one with two genomes, and the other with 143 genomes (Potter et al., 2018). The genomes in the second lineage formed 26 clusters, with variations in virulence and infection severity between different strains, but no correlation was observed between cluster and geography, niche, or virulence level. A mosaic distribution of virulence-related genes has been observed among clinical and plant-associated *K. variicola* strains (Martínez-Romero et al., 2017b), but when considering the possibility that some genes may be phenotypically silent, it is difficult to draw meaningful conclusions without sufficient *in vitro* and *in vivo* data.

The behavior and niche of these microbes are also impacted by their metabolic profiles. Nitrogen fixation, for example, is a metabolic capability that strongly affects niche preference. In contrast to *K. pneumoniae*, where nitrogen fixation genes are located in the accessory genome and encountered infrequently, nitrogen fixation is a hallmark of *K. variicola*, with the requisite *nif* genes contained in its highly conserved core genome (Martin and Bachman, 2018). Indeed, of 294 *K. pneumoniae* genomes analyzed in a global survey, only one contained the *nif* operon (Holt et al., 2015), and it has been proposed that the absence of nitrogen-fixation capacity contributed to *K. pneumoniae*'s evolution toward human hosts and its increased pathogenicity compared to its consistently nitrogen-fixing plant-associated relative, *K. variicola* (Fouts et al., 2008; Brisse et al., 2014). One *K. variicola* outlier strain, NL49/58, acquired the capability to ferment adonitol – a metabolic characteristic typically present in other *Klebsiella* species such as *K. aerogenes*, *K. planticola*, *K. oxytoca*, and *K.*

pneumoniae (Brisse and Verhoef, 2001; Davidson et al., 2015). This trait likely rendered NL49/58 capable of colonizing milk, underlying its reported link to cases of bovine mastitis, which had previously been associated with *K. pneumoniae* and *K. oxytoca* (Podder et al., 2014; Masse et al., 2020).

Taken together, these studies highlight the importance of delineating genotypic and phenotypic differences not only between *K. variicola* and other *Klebsiella* species, but also between different *K. variicola* strains. This is a critical step in the process of evaluating the safety of this highly heterogeneous species, and should include not only *in silico* screening, but also *in vitro* and *in vivo* characterization of pathogenicity potential in plants and animals.

Beneficial environmental *Klebsiella variicola* strains

Environmental *K. variicola* strains are of special interest for their potential uses in sustainable agriculture and industry (Duran-Bedolla et al., 2021). Drawn to nutrient-rich root exudates (Feng et al., 2021), plant-associated strains exist as endophytic or rhizospheric nitrogen-fixing symbionts, and provide their hosts with a bioavailable source of nitrogen and increased resilience toward environmental stressors (Souza et al., 2015; Vives-Peris et al., 2020). Examples of such strains abound in the literature, and *K. variicola* has been isolated from and shown to support the growth of numerous crops, including sugarcane, soybeans, maize, tomatoes, and peanuts (Wei et al., 2013; Kim et al., 2017; Yang and Yang, 2020; Guerrieri et al., 2021; Qin et al., 2022; Wang, 2022; Zhang et al., 2022).

The reported effects of *K. variicola* on its plant hosts are notable; a greenhouse study in sugarcane reported a 79% increase in whole-plant nitrogen content and 51% greater whole-plant dry biomass with *K. variicola* inoculation (Wei et al., 2013), and studies in tomato plants showed significant increases in yield and above-ground biomass (Guerrieri et al., 2021; Nava-Faustino et al., 2022). It has

been reported to be a highly conserved core member of the nitrogen-fixing microbial community in maize plant xylem – a finding that was consistent across substantially different soil types, fertilization regimes, and soil microbial communities analyzed in the study (Zhang et al., 2022). It has also been shown to mitigate flood stress in soybeans (Kim et al., 2017), and improves salinity stress tolerance in wheat, maize, and tuberose (Ghazi et al., 2021; Kusale et al., 2021a, 2021b). Isolated instances of plant pathogenicity have also been reported, primarily in tropical monsoon climates: banana rhizome rot, carrot soft rot, and sorghum leaf streak in India (Chandrashekar et al., 2018; Loganathan et al., 2021; Malik, 2023), banana soft rot in China (Fan, 2016), and plantain soft rot in Haiti (Fulton et al., 2020). However, it should be noted that these plant pathogenicity reports rely on intrinsically limited culture-based isolation and detection methods. Additional field trials are needed to identify all relevant species, both cultivable and uncultivable, to recapitulate pathogenicity under conditions consistent with the natural environments of these crops, and to validate plant beneficial activity and elucidate mechanisms. Meanwhile, selected strains have been successfully harnessed as biofertilizers for cereal crops, and *K. variicola*'s utility in providing a stable, safe, and sustainable nitrogen source has now been demonstrated commercially on millions of farm acres (Wen et al., 2021; Arbaugh et al., 2022; Temme, 2022).

K. variicola soil isolates have also been identified and studied for use in the remediation of soil and industrial waste. Several strains tolerant to toxic halides, heavy metals, and aromatic compounds show efficacy in the treatment of wastewater, industrial effluents, and contaminated soil. For example, a strain isolated in Bangladesh effectively reduced highly toxic chromium (VI) produced by leather tanning, a major source of industrial pollution, and showed a high tolerance to cadmium, zinc, nickel, and mercury (Kabir et al., 2018). When loaded onto biochar, another strain removed up to 96% of chromium (VI) from groundwater samples collected near a leather tannery in China (Yu et al., 2021). Another biochar system, in this case loaded with a bacterial consortium including *K. variicola* and two *Enterobacter* species, reduced cadmium levels in contaminated soil, reduced its levels in bok choy plants by up to 50%, and simultaneously increased dry shoot biomass of the plants by an average of 240% (Wei et al., 2022). Strains with bioremediation activity against toxic aromatic compounds (Liu et al., 2016), lead (Das and Osborne, 2017), red azo dye (Eslami et al., 2019), and atrazine (Pan et al., 2023) have also been reported. Future studies are needed to further validate these findings, but reports to date clearly indicate *K. variicola*'s potential for the remediation of a range of toxic waste and pollutants, provided the right strains are selected, optimized, and applied to the specific niche and context in which they're intended to function.

Clinical strains of *Klebsiella variicola*: direct drivers of infection, or opportunistic passengers?

The emergence of enhanced diagnostic techniques has enabled several large-scale population structure studies of the KpSC (Berry et al., 2015; Long et al., 2017; Martínez-Romero et al., 2017a;

Ohama, 2022). These studies show that (1) *K. variicola* accounts for only 2-6% of KpSC-positive human samples, and (2) pathogenicity is not clearly associated with lineage for these species, but rather linked to specific virulence factors, which are found with consistently lower frequency in *K. variicola* (Holt et al., 2015; Lam et al., 2021; Dereeper et al., 2022; Morgado et al., 2022). Phylogenetic analyses of *gyrA*, *parC*, and *rpoB* gene sequences have categorized *K. variicola* as a distinct phylogroup of KpSC, specifically KpIII (Brisse and Verhoef, 2001; Brisse et al., 2004; Garza-Ramos et al., 2015), while *K. pneumoniae* (KpI) is by far the dominant agent of *Klebsiella*-associated human infections (Pendleton et al., 2013; Lam et al., 2021). Furthermore, a global population analysis of over 11,000 *Klebsiella* genomes showed an extremely low prevalence of both virulence and antimicrobial resistance factors for *K. variicola*, with the exception of β -lactamase resistance, which is a chromosomally encoded feature of all KpSC species (Lam et al., 2021). *K. variicola* isolates associated with human infection, primarily isolated from blood, urine, and sputum, have been reported in immunosuppressed (e.g., cancer and trauma patients) and immunocompromised (e.g., diabetics, the elderly, neonates) populations (Maatallah et al., 2014; Long et al., 2017; Farzana et al., 2019; Imai et al., 2019; Piepenbrock et al., 2020; Kiley et al., 2021; Watanabe et al., 2022). Both retrospective and case studies underscore the clear opportunistic nature of this commensal bacterium, describing the infection of individuals with weakened immunity or comorbidities. Several studies also show evidence that a major source of KpSC infections in hospitalized patients may in fact be the patients' own microbiota opportunistically spreading or overgrowing under conditions of weakened immunity (Martin et al., 2016; Gorrie et al., 2017, 2018).

One retrospective analysis of bloodstream infections (BSI) in Norway has suggested that *K. variicola* carries a higher 30-day mortality rate compared to *K. pneumoniae* (Maatallah et al., 2014); however, this conclusion is based on a single study with considerable limitations. The study was limited to one site, had a relatively small sample size (139 KpSC cases, 34 of which were *K. variicola* infections), and lacked sufficient biological and clinical relevance to inform practice or draw solid conclusions. BSI are generally considered to be a marker of severe primary disease irrespective of the causative agent, and present a poor prognosis and high mortality rate (Goto and Al-Hasan, 2013). Among the BSI patients included in this study, *K. pneumoniae* remained the most frequent causative agent (69%). Of the 10 *K. variicola* infected patients suffering 30-day mortality, all 10 presented comorbidities, and their average age was 75. Moreover, mortalities in the *K. variicola* group were not linked to any known virulence factors. While this study does not provide sufficient evidence to make meaningful risk determinations or impact patient treatments or outcomes, it does highlight, yet again, the prerequisite of weakened immunity for successful *K. variicola* infection.

A contextualized risk assessment paradigm

The demand for safer and more sustainable agricultural inputs has increased tremendously in recent years, and industry is

responding accordingly. The global biofertilizer market, valued at \$1.8 billion USD in 2021, is projected to grow to \$4.4 billion by 2029 (Biofertilizer Market Size, 2021). Significant technological advances have enabled agricultural companies to develop a wide array of microbial products, all of which require appropriate safety evaluation. Given the recognized nuance and complexity of host-microbe interactions and the significant differences between strains within a species, strains must be evaluated individually, with a focus on their intended application and usage parameters. As described above, multiple beneficial activities of *Klebsiella variicola* in environmental settings have been reported. Evaluation of this highly heterogeneous species could encompass several key considerations, including, but not limited to, a strain's genotype and phenotype (including virulence and antimicrobial resistance factors), *in vivo* toxicity, virulence and non-target effects, and potential routes of human and environmental exposure based upon intended application methods.

In the case of *K. variicola* strains developed for use as biofertilizers, the intended context is exclusively environmental, by way of application to crops. Revisiting the question of safety in light of this context leads to several observations. First, studies to date do not indicate infection risks in healthy humans, and virulence and antibiotic resistance factors, already found infrequently in *K. variicola*, would not encounter the acquisition pressures found in a hospital or community environment when applied in the field. Furthermore, these strains are not intended for direct human exposure, and application methods are designed to minimize exposure risk. Strains selected for field use were well characterized in terms of mechanism of action, genotype, and phenotype, and did not show toxicity or pathogenicity in a standard battery of *in vivo* biosafety tests (Wen et al., 2021). Finally, given that *K. variicola* is a ubiquitous soil microbe and plant symbiont found around the world, its presence in agricultural environments is not novel.

Conclusion

The concept of bacterial risk is nuanced, dynamic, and highly dependent on context. As evidenced increasingly in human microbiome studies, the lines between commensal, mutualistic, and pathogenic bacteria are often hazy. Whether a host-microbe interaction is beneficial, detrimental, or neutral depends on numerous factors, including a bacterial strain's genotype, fitness, and niche, as well as host health, degree of exposure, and the prevailing microbial environment. *Klebsiella variicola* is a near ubiquitous diazotroph found primarily in soil and plants, and has tremendous potential as a biofertilizer. It is also a frequent resident of the human microbiome, and clinical reports do not indicate a risk of pathogenicity in healthy individuals. Moreover, *K. variicola* strains developed for biofertilizer use are intended strictly for targeted application in select crops, and are chosen and optimized for the selective colonization of plants over other hosts. With these considerations in mind, we find no indication that the judicious

application of *K. variicola* strains optimized for use as biofertilizers in select crops presents undue safety concerns to healthy humans or the environment. The context-specific approach presented herein illustrates a critical paradigm shift in the microbial risk evaluation process – a necessary step in creating a path forward for the development, commercialization, and safe use of microbial products across multiple industries.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ChM: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. FaS: Writing – review & editing, Supervision, Conceptualization. CaM: Writing – review & editing, Conceptualization. FoS: Writing – review & editing, Supervision, Conceptualization. NH: Writing – review & editing, Supervision, Conceptualization, Project administration, Resources. NR: Writing – original draft, Data curation, Methodology, Visualization. NV: Writing – review & editing. ES: Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We are grateful to Nicholas Colaianni for providing technical guidance on screening the Human Microbiome Project database, Chris Cornelius for formatting figures, and Sophie Kowaliczko for assistance with reviewing literature. We also thank Allen Kuo for providing legal insight and contract preparation, and Mitchell Craft, Wendy Watkins, and Mary Burich for manuscript review.

Conflict of interest

Authors ChM, FaS, CaM, FoS, NH and NR were employed by the company Pivot Bio. Pivot Bio has filed patent applications and is developing and commercializing products based on microbial strains presented in this manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/finmi.2024.1412302/full#supplementary-material>

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