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Microbial ecology of cereal vinegar fermentation: insights for driving the ecosystem function

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Over thousands of years, humans have mastered the natural vinegar fermentation technique of cultivating functional microbiota on different raw materials. Functional microbial communities that form reproducibly on non-autoclaved raw materials through repeated batch acetic acid fermentation underpin the flavour development of traditional cereal vinegars. However, how to modulate rationally and optimise the metabolic function of these naturally engineered acidic ecosystems remains unclear. Exploring two key minorities in a vinegar ecosystem, including microbial functions (e.g., flavour and aroma synthesis) and microbial strains, is a crucial step for the vinegar industry to modulate the metabolic function of vinegar microbiota, to monitor the fermentation process, and to maintain the flavour quality of final product.

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Introduction

Over thousands of years, humans have mastered the natural vinegar fermentation technique of cultivating functional microbiota on different raw materials (e.g., vegetables, fruits, and animals), for degrading substrates and developing flavours [1]. Manipulation of environmental variables such as nutrient, humidity, temperature, and aeration results in a wide spectrum of fermented vinegars [2]. In East Asia, especially in China, cereals are

commonly used for the production of vinegars with characteristic flavours [3]. Metabolism of various microorganisms in the vinegar fermentation improves the nutritional value, sensory properties, and bioactivities of raw materials [4].

Traditional cereal vinegars are typically fermented by solid-state or semi-solid-state fermentation techniques via three major steps: starch saccharification, alcohol fermentation, and acetic acid fermentation (AAF) [5]. Under the influence of fermentation starter-*Jiuqu*, cereal starch is enzymatically converted to fermentable sugars, which is further transformed into ethanol. Alcohol mash is mixed with wheat bran, rice hull, and vinegar starter in different pots or ponds, and microbes that inhabit solid-state vinegar culture (termed *Pei* in Chinese) synthesise an abundant quantity of acids as well as various flavours in ~20 days of AAF. In a recent study, a total of 360 volatile compounds have been identified from Zhenjiang aromatic vinegar, a representative Chinese cereal vinegar with sticky rice as the main material [6]. By comparison to the cereal vinegar production, no saccharification step exists in the production of many sugar-rich fruit vinegars, such as traditional balsamic vinegar. Meanwhile, the acetic acid fermentation of fruit vinegars is usually performed in liquid state or semi-solid-state by inoculating special strains belonging to acetic acid bacteria [1].

Traditional cereal vinegar production is a spontaneous mixed-culture process that has been performed for many centuries without spoilage. Although the fermentation is carried out with non-autoclaved raw materials (e.g., sorghum, sticky rice, and wheat bran) in an open work environment, the pattern of microbial community succession and flavour formation remains uniform from batch to batch [7]. From an ecological perspective, an acidic vinegar culture (pH 3.0) in the repeated batch AAF is a stressful but reproducible ecosystem, which provides an opportunity for dissecting the mechanisms underlying the microbial community formation and function [8,9••]. Over thousands of years, craftsmen have optimised the fermentation conditions that promote the growth of microbes in the vinegar microbiota based on practical experience. However, a scientifically based understanding of the changes in the microbial community remains elusive. Recently, there is an increasing interest to rationally optimise and modulate the metabolic function of this naturally engineered ecosystem. Meanwhile, it is increasingly important for the vinegar industry to control

the efficiency of fermentation, and to maintain the product flavour intensity.

In order to modulate the metabolic function of a microbial complex system, we must investigate the pattern and functionality of the microbial community, the interaction between individual species, and the dynamic response to environmental influences of the microbes that inhabit these naturally engineered vinegar ecosystems. The aim of this review is to present a survey of recent investigations on the assembly and function of the microbial community in traditional cereal vinegar fermentation, and discuss the approaches to drive the metabolic function of vinegar microbiota.

Assembly of cereal vinegar microbiota
Microorganisms from a variety of resources including non-autoclaved cereals [10], *Jiuqu* [11], vinegar *Pei* starter [7], and the open work environment account for the diversity of the cereal vinegar microbiota. Li *et al.* [12•] have reviewed previous studies on isolated strains from different vinegar fermentation processes. Acetic acid bacteria (*Acetobacter*, *Gluconobacter*, *Gluconacetobacter*), lactic acid bacteria (*Lactobacillus*, *Pediococcus*), and *Bacillus* are the main genera easily isolated in the AAF of cereal vinegars [13–15]. Recent applications of culture-independent approaches including amplicon-based high-throughput sequencing and PCR-denaturing gradient gel electrophoresis (DGGE) analysis have provided a blueprint of microbial diversity in the vinegar ecosystems for Zhenjiang aromatic vinegar, Shanxi aged vinegar, and Tianjin duliu mature vinegar [16–21]. A picture that includes 951 genera within the vinegar *Pei* of Zhenjiang aromatic vinegar has been unravelled by metagenomic sequencing [22]. The diversity of these vinegar microbiota might have been underestimated because the existing taxonomic annotations of metagenomic reads are often incomplete. As the reference databases such as Genbank and KEGG improve, the taxonomic assignment of metagenomic reads will become more accurate [23].

Although countless microbes enter the AAF systems, *Lactobacillus* and *Acetobacillus* dominate the bacterial communities. A community-level microbial self-domestication process has been observed through the AAF processes of Zhenjiang aromatic vinegar, Shanxi aged vinegar, and Tianjin duliu mature vinegar [16,19–21]. Many environmental conditions in the cereal vinegar ecosystem, such as high alcohol content in the alcohol mash (9%), the elevated acidity (pH 3.0) in vinegar *Pei*, coexistence of both the aerobic and the anaerobic environments, and the bio-heat ($\sim$45 °C) maintained in vinegar *Pei*, might correlate to the growth of microbes and help to maintain a consistent microbial community and flavour formation over repeated batch cycles [24]. Yet, the key drivers of these patterns and microbial population fidelity to specific environmental conditions remain to be determined. It is important that future studies disentangle microbial community interactions in the vinegar microbiota by using statistical and bioinformatic tools such as a metagenomic microbial interaction simulator (MetaMIS) [25,26].

Function of cereal vinegar microbiota
Cereal vinegar microbiotas are often enormously diverse. Moreover, the interaction among community members and that between the members and the abiotic/biotic environment are extremely complex. Therefore, understanding the function of the cereal vinegar microbiotas is quite a challenge. Wang *et al.* [19] have used bidirectional orthogonal partial least squares (O2PLS)-based correlation analysis to connect the data matrices of microbiota succession and flavour dynamics through the AAF of Zhenjiang aromatic vinegar. Seven genera including *Acetobacter*, *Lactobacillus*, *Enhydrobacter*, *Lactococcus*, *Gluconacetobacter*, *Bacillus*, and *Staphylococcus* were predicted as the few key microbes for the production of major vinegar flavours. Statistical approach provides a perspective for bridging the gap between the phenotype and the genotype of a vinegar ecosystem. However, the metabolic characteristics of these functional microbes in a community context require wet-lab experiment validation.

Another challenge in dissecting the metabolic roles of microorganisms in a vinegar ecosystem is the reconstruction of microbial metabolic interaction networks [27]. Wu *et al.* [22] have elucidated the flavour metabolic network in the microbiota of Zhenjiang aromatic vinegar, and revealed the distribution discrepancy of microorganisms in different metabolic pathways. Via this approach, besides the microbes responsible for organic acids metabolism, two important functional microbial groups participating in the biosynthesis of acetoin-diacetyl-2,3-butanediol and 3-methylbutanol-3-methylbutanoic acid-leucine-valine were discovered. The results verified the functional complementation of microbes in a vinegar ecosystem. Because the metabolic function of microbes evolves with fermentation time, the succession of a vinegar microbiota function should be explored in the future.

Modulation of the metabolic function of vinegar microbiota
The metabolic function of a vinegar microbiota can be modulated by adjusting fermentation parameters and by inoculating with pre-enriched functional strains in suitable proportions. Here, we present a generally applicable approach of vinegar bioaugmentation in three steps: firstly, analysing the assembly and function of the vinegar microbiota; secondly, culturing the key microbial minority-the constitutive functional strains from fermented cereals based on the predicted metabolic information; and thirdly, evaluating the metabolic function of the isolated constitutive strains *in situ* and *ex situ* and