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COMPARATIVE ANALYSIS OF YEAST IDENTIFICATION METHODS IN TRADITIONAL FERMENTED BEVERAGES: PHENOTYPIC, PROTEOMIC, AND GENOMIC APPROACHES

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ABSTRACT

Traditional fermented beverages are a value in the global artisanal market huge repository of microbial diversity of historical importance, which are underpinned by complex and interacting communities of yeast and bacteria. The exact taxonomic identification of these yeasts is not only an academic task but is also a key step to understanding the ecology of fermentation, the improvement of industrial starter cultures, the safety of the products in the presence of more virulent spoilage organisms and the maintenance of distinctive organoleptic properties of local fermented products. This review report critically discusses the evolution and the status of yeast identification methods, including the traditional culture-based method and the old nucleic acid extraction procedures, as well as new and emerging automated systems. A detailed comparative study is performed between the bioMérieux VITEK 2 Compact system and the bioMérieux RapID Yeast Plus system and high-resolution genotypic techniques such as ITS and D1/D2 domain sequencing that can distinguish cryptic species complexes. In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is thoroughly explored as a disruptive and paradigm shifting proteomic technology. This report shows that, in spite of the potential of automated phenotypic systems for fast clinical diagnosis, the value of these systems is severely restricted in the case of the environmentally stressed, highly phenotypic yeast strains typical of traditional fermented drinks. Finally, a polyphasic taxonomic strategy combining genomic accuracy with the proteomic rapidity and biochemical profiling is established as the gold standard for yeast robust characterization in food and beverage microbiology.

Keywords: *Yeast Identification*[1], *Traditional Fermented Beverages*[2], *Phenotypic Characterization*[3], *Proteomic and Genomic Approaches*[4], *Molecular Microbiology*[5].

1. INTRODUCTION

Controlled microbial fermentation is one of the oldest and most enduring biotechnological accomplishments of mankind. In the millennia that have passed, various cultures have used different spontaneous microbial consortia to enhance the biochemistry and flavour of raw agricultural substrates, from cereals to fruits, palm sap to mammalian milk. In addition to being important methods of preserving and improving food in the developing world, these native products of fermentation are also important cultural treasures and economic assets in the international artisanal market as described in the seminal Handbook of Indigenous Fermented Foods (Steinkraus, 1995).[1] [2], [3] In recent years, the importance of these traditional drinks as health-promoting has been highlighted more and more in epidemiological and nutritional analysis. Water kefir, fermented milks, and African drinks derived from cereal grains can all be used as delivery matrixes containing probiotic microorganisms. However, the therapeutic properties, microbiological safety and sensory quality of these drinks are completely dependent on the composition and metabolic activity of the microbial ecology that they carry within them [4], [5],[6], [7].

These intricate microbial communities rely on yeasts as their main drivers of alcoholic fermentation and secondary metabolite generation. There are methodologic problems in the reliable identification of these yeasts. Because of the complexity of the taxonomy of the fungal kingdom, and the plastic appearance of the yeasts under the extreme conditions imposed by fermentation (high ethanol concentration, low pH, osmotic shock, temperature) the traditional identification methods are not applicable. This report thoroughly examines the effectiveness of modern platforms for yeast identification, and offers a scientifically sound summary of the phenotypic, genotypic and proteomic approaches to create a clear-cut classification for yeast taxonomy in the field of food science.[8], [9], [10], [11], [12], [13], [14]

2. MICROBIAL ECOLOGY AND SUBSTRATE ADAPTATION IN TRADITIONAL FERMENTED BEVERAGES

Geographical Diversity and Beverage Matrices: Traditional fermented beverages are remarkably diverse throughout the world as far as their microbial populations and substrates. The type of yeast used in any particular alcoholic drink is closely related to the type of raw agricultural products used in the drink, local climate, and traditional production techniques. They often require back-slopping, or the addition of a successful fermentation batch, and/or complex, dehydrated solid-state starters. [4], [6], [15], [16], [17], [18], [19] Naturally, cereal drinks are mainly found in Africa, where they are both rich in and complex with respect to nature. In these particular regions, the yeast populations have to be adapted to grow in a strongly acid environment created by co-fermentation with lactic acid bacteria (LAB) like *Lactobacillus species*, *Leuconostoc species* and other lactic acid bacteria.[4], [6], [19], [20], [21], [22]. Consortia, which are complex communities of microorganisms, are traditionally used in Asia and Eastern Europe and are usually visible to the naked eye. The kefir grains are from the Caucasus region and contain a complex polysaccharide matrix, a symbiotic, self-perpetuating community of yeasts, LAB and acetic acid bacteria, as is the case with the rice-based beverages.[1], [4], [17], [23], [24], [25]

Yeast spoilage: A threat in beverage matrices: Despite the emphasis of food microbiology on the positive applications of fermentative yeasts, proper identification is also essential for the strict quality control and to avoid the disastrous low economic values resulting from spoilage. Yeasts are present in the agricultural setting and are a common contaminant in food and beverage processing operations even when they take care of sanitation.[26], [27], [28], [29] The yeast's spoilage is described by Fleet (2011) as being a range of undesirable organoleptic changes which may be manifested, and as the cold chain storage only delays the appearance of the spoilage symptoms, there is no way to prevent its occurrence. [10], [30] The genus *Zygosaccharomyces* is a group of organisms that are highly tolerant of high sugar concentrations and are known as highly osmotolerant spoilage agents especially in the fruit and beverage industry, where they grow in the high sugar content of fruit concentrates and syrups. Moreover, they are known to be highly resistant to weak-acid preservatives, including sorbic and benzoic acids, which are commonly used throughout the beverage industry worldwide, making identification methods that have a high resolution of identification and distinguish closely related taxa an absolute requirement for Hazard Analysis Critical Control Point (HACCP) programs to trace spoilage vectors and target sanitation steps at a species-specific level.[10], [26], [30], [31], [32], [33], [34]

3. FUNCTIONAL CONTRIBUTIONS: EXTRACELLULAR HYDROLYTIC ENZYMES AND AROMATIC PRECURSORS

Saccharomyces cerevisiae is well established as the leading fermentative organism used in a wide range of alcoholic drinks because it is unsurpassed in its ability to withstand the presence of ethanol and to rapidly produce glycolytic fluxes. The focus of microbiological surveys, however, has been radically changed by modern trends, which have turned to the essential biochemical role of non-Saccharomyces yeast. Traditional brewers and wine makers used to consider genera like *Candida, Pichia, Rhodotorula, Hanseniaspora* and *Kluyveromyces* to be contaminants in the early stages of alcoholic fermentation, but we now know that they are key drivers of flavours, aromas and structural characteristics in beverages. The enzymatic potential of the sample was screened exhaustively. The sample was exhaustively screened for enzymatic potential.[8], [9], [35], [36], [37], [38], [39], [40].

Exhaustive Screening of Enzymatic Potential: These non-*Saccharomyces* species are mainly significant for their extensive enzyme activities. In the first, low-ethanol stages of fermentation these yeasts release a wide variety of extracellular hydrolytic enzymes (EHE) into the beverage matrix. This large scale screening, which involved a broad spectrum of yeast species from four genera (*Kloeckera, Candida, Debaryomyces, Rhodotorula*) and nine genera (*Pichia, Zygosaccharomyces, Hanseniaspora, Kluyveromyces*) of yeasts, was a systematic evaluation of 245 isolates from vineyards and clarified juices by Strauss et al. (2001).[8], [9], [35], [38], [39], [41]. The methods for detection of these enzymes were very specific: For example, the activity of xylanase was detected on special agar plates containing xylan, and the sulfite reductase activity was detected by plating the yeasts on a solid juice indicator agar supplemented with bismuth citrate, a bismuth sulfide precipitate being observed to confirm the activity of the enzyme. In addition, β-glucanase activity was confirmed by Congo red staining on Yeast Beta Glucan supplemented media, where clear degradation halos were observed. [35], [42], [43], [44], [45]. The study showed that all the enzymes screened were expressed in a very good way across all the genera, a fact that is very important during fermentation: pectinases and β-glucanases break down complex structural polysaccharides and clarify the beverage, which also helps with the filtration process;

proteases release assimilable nitrogen that is important for the healthy fermentation kinetics of *S. cerevisiae*.[35], [39], [40], [41], [44], [46]

Critical Absence of Beta-Glucosidase Activity: This complete, systematic absence of extracellular β-glucosidase activity is however a very significant biochemical finding in this exhaustive screening of 245 isolates. For example, in certain fermentations using grapes or fruits, important aromatic substances like monoterpenes, benzene derivatives, and nor-isoprenoids can be chemically bound to sugar molecules (glycosides), making them odourless and non-volatile.[35], [47], [48], [49], [50], [51], [52] The lack of this enzyme in many of the indigenous wild yeasts established is of interest for industrial processes, because it indicates that naturally occurring, spontaneous fermentations may not be capable of realizing the full aromatic potential of the raw material. It highlights the absolute need of precise yeast strain identification and molecular characterization during bio-prospecting to find new enzyme-producing starter cultures that can catalyze the desired, specific biotransformation(s).[9], [39], [47], [49], [53], [54], [55]

4. THE ERA OF PHENOTYPIC CHARACTERIZATION AND CLASSICAL ISOLATION

Previously, the isolation, classification and identification of yeasts from complex beverage matrices was done solely by culture-dependent methods and classical biochemistry. The basic procedure is serial dilution of the beverage followed by spreading of the diluted samples on selective agars.[10], [33], [56], [57]

Historical Context of Yeast Nucleic Acid and Protoplast Extraction: Prior to the advent of modern genomics, basic biochemical techniques for the extraction of cellular components needed to be developed to conduct yeast identification. The yeasts have a very stable and resistant cell wall, which is constructed primarily from interwoven β-1,3-glucans, β-1,6-glucans, chitin, and heavily glycosylated mannoproteins, unlike many bacteria. This matrix poses a big problem in cell lysis and subsequent extraction of intact DNA. Though a successful methodology, one that eventually became a blueprint for genomic profiling, K.S. Kirby's acid phenol extraction worked with the highly corrosive and toxic phenol, and was labor intensive, requiring multiple rounds of extraction to obtain adequate purity of the nucleic acids.[11], [58], [59], [60], [61], [62], [63], [64] At the same time, work was undertaken to find ways to escape the yeast cell wall completely in order to carry out physiological studies. The methods of Eddy and Williamson (1957) were the precursors of methods for the isolation of protoplasts and the subsequent DNA extraction methods that have enabled the development of high-resolution molecular taxonomy today.[10], [64], [65], [66], [67]

Limitations of Classical Biochemical Assays: After successful isolation, a complete set of manual biochemical tests would traditionally have been used to make a definitive phenotypic identification. The assays tested a yeast's urease activity and its ability to grow on a variety of carbon and nitrogen sources in both aerobic and anaerobic conditions, ferment various carbohydrates, and grow over a range of temperature and osmotic gradients [10], [11], [33], [56]. Classical phenotypic characterization has a significant operational inefficiency as its main drawback. It takes up to 10-14 days to get a complete diagnostic profile. In addition, there is considerable subjectivity in manual reading of turbidity in assimilation broths. The most important part of this is that the strains that are isolated from the fermented beverages often have atypical biochemical profiles, or phenotypic plasticity, which is caused by metabolism being down regulated as a result of the harsh environment of the fermentation matrix. This ecological stress results in frequent misidentifications in reference to standardised taxonomic keys.[10], [11], [33], [56], [68], [69], [70]

5. AUTOMATED BIOCHEMICAL PROFILING: DEEP DIVE INTO THE VITEK 2 COMPACT SYSTEM

In order to address the issues of subjectivity and high labor requirement of the manual biochemical tests, automation of microbial identification platforms was developed, and the bioMérieux VITEK 2 became one of the most important technologies in clinical and industrial microbiology laboratories.

System Architecture and the ID-YST Card: The VITEK 2 system is an automated phenotypic identification system, based on miniaturized, self-contained identification cards. The ID-YST card is a very sophisticated plastic substrate with 64 individual microwells that contain 47 individual, dried biochemical substrates which can produce a specific metabolic reaction to the organism cultured in the wells.[71], [72] The biochemical profile measured with the ID-YST card is very complete. It comprises 20 specific carbohydrate assimilation reactions such as adonitol (ribitol), D-trehalose, D-cellobiose, dulcitol, D-galactose, D-glucose, lactose, D-maltose, D-mannitol, D-melibiose, D-melezitose, and palatinose. Also the card evaluates the assimilation of six distinct organic acids: N-acetyl-glucosamine, methyl-α-D-glucopyranoside, citrate, D-galacturonate, D-gluconate, and mono-methyl-ester-succinate.[70], [71], [73], [74], [75]

Fluorometric Principles and Kinetic Analysis: The VITEK 2 system is a major step from the traditional, often inaccurate colorimetric reading of the past to a highly sensitive fluorometric detection system. Eight specific substrates designed for the detection of oxidases are covalently coupled with the highly fluorescent molecule 4-methylumbelliferone (4MU).[19] These include complex substrates such as α -galactoside-4MU, α -glucoside-4MU, α -mannoside-4MU, β -galactoside-4MU, β -glucoside-4MU, and β -glucuronide-4MU.[71], [72], [75], [76], [77], [78] .When a specific yeast enzyme cleaves the target substrate, the 4MU fluorophore is chemically liberated. The machine's internal optics excite the free 4MU to produce a fluorescent signal that is precisely measurable. High degree of standardization in the operational workflow. The ID-YST card is sealed after being automatically filled in a vacuum by the VITEK 2 instrument and is transferred to a sub-vacuum inside an incubator, which is maintained accurately at 35.5 °C, within the instrument. [71], [73] This continuous monitoring dramatically shortens diagnostic time by subjecting the cards to a fluorescence measurement every 15 minutes. The computerized system automatically calculates the final identification profile after only 15 to 18 hours of incubation, compared to weeks needed for manual testing; this profile is then compared to an ID-YST database stored in a proprietary software program, and the identification is subsequently categorized as “excellent”, “very good”, “good”, “acceptable”, “low discrimination” or “unidentified”.[70], [71], [72], [73], [75], [78]

6. CRITICAL EVALUATION OF VITEK 2 PERFORMANCE METRICS

The architectural design of the VITEK 2 is impressive, but the empirical performance is highly dependent on the taxonomic predictability and ecological origin of the yeast to be determined.

Clinical Efficacy vs. Taxonomic Blind Spots: Pincus et al. (2000) showed the system to be highly effective in a basic multicenter test in which a culture of standard isolates was used. In all, 222 (92.1%) strains were correctly identified by the Vitek 2 directly to the species level, with 11 strains (4.6%) being poorly discriminated but readily solved using simple additional tests.[71], [75], [78], [79] The system did, however, seem to have some clear and mechanically intrinsic limitations with respect to certain taxonomic boundaries. The performance summary is presented in Table 1 below:

Table 1: Performance metrics of the VITEK 2 ID-YST system in identifying yeast and yeast-like organisms, adapted from Pincus et al. (2000). [71]

Identification Category	Number of Strains	Percentage of Total (n=241)
Unequivocal Identification	222	92.1%
Low Discrimination (Resolved)	(11)	(4.6%)
Low Discrimination (Unresolved)	10	4.1%
Misidentified	4	1.7%
Unidentified (Unknown Profile)	5	2.1%

A more thorough examination of the weaknesses shows important systemic weaknesses. Pincus et al. (2000) reported that the failure was not caused by either false-positive or false-negative machine errors as there were no errors in this instance, but rather the strains were not sufficiently discriminated against. Manual supplemental tests, such as cellobiose assimilation (Wickerham method) should also be performed, but the VITEK 2 system cannot manually perform the necessary tests to differentiate these two closely related species.[71] In addition, the system had a very specific misidentification pattern for Candida krusei. Multiple cases of misidentified C. krusei strains have been reported, as the Vitek 2 system has falsely suggested the identification of the strain as Geotrichum capitatum because of the atypical behavior of some strains in biochemical reactions, and the overlapping phenotypic parameters that led to the automated conclusion.[70], [71], [79], [80], [81]

Comparative Analysis with RapID Yeast Plus: An important aspect of the limitations of biochemical automation is highlighted by comparing several platforms. The VITEK 2 system was evaluated in addition to the RapID Yeast Plus system in a large study of 750 yeast isolates. The RapID system correctly identified 716 isolates (95.5%), misidentified 18 isolates (2.4%) and failed to identify 16 isolates (2.1%)[80]. What is important is that 24 specific isolates were incorrectly identified by both commercial systems. Predictable biochemical changes also occur in species such as Candida parapsilosis, where one isolate was misidentified as Candida famata by the Vitek 2 system and Candida lambica by the RapID Yeast Plus system; and in Candida krusei, where a single isolate was misidentified as Candida lipolytica by both systems. These unpredictable biochemical differences make the use of automated phenotypic systems suboptimal as definitive identification tools for the mapping of biodiversity in the context of environmental isolates from fermented beverages, which are exposed to very high levels of acid stress and ethanol toxicity.[70], [80], [81], [82]

7. THE MOLECULAR TAXONOMY PARADIGM: GENOMIC SEQUENCING AND MARKER ANALYSIS

The intrinsic limitations of phenotypic plasticity, the absence of specific substrates, and a limited clinical database resulted in a paradigm shift in the world of yeast taxonomy towards molecular genotyping. Unlike other diagnostic methods, molecular techniques are unaffected by environmental factors or culture requirements and are able to directly analyse highly conserved, but strategically variable sequences, of the fungal genome.

Ribosomal DNA (rDNA) as Taxonomic Barcodes: Most of the modern molecular taxonomy of yeasts is based on the architecture of the cluster of genes coding for the fungal ribosomal RNA (rRNA). There are several copies of this genomic cassette in the genome to provide a abundant number of templates for Polymerase Chain Reaction (PCR) amplification. The D1/D2 region (differential domain) at the 5' end of the large subunit (LSU) 26S (or 28S) rRNA gene is a very critical diagnostic region and amplification of the full D1/D2 region generates an approximately 600 base pair (bp) sequence that can be interrogated with a robust public repository, such as the GenBank database, to provide very accurate species-level identification of nearly all known ascomycetous and basidiomycetous yeast species, especially to validate ITS results, and also to resolve specific taxonomic clades where ITS interspecific divergence is anomalously low. Comparative studies have shown identification by D1/D2 and ITS sequence analysis to agree nearly perfectly in all cases, making them the gold standard of identification.[12], [80], [83], [84], [85], [86], [87]

Resolving Cryptic Species Complexes: The *Candida Parapsilosis* Case

The molecular sequencing approach really shines when compared with the phenotypic approach in the ability to resolve "cryptic species complexes," which are groups of yeast species that are indistinguishable in terms of morphology and biochemistry. One of the classic examples of this in mycological literature is the Candida parapsilosis complex. Isolates identified as this species, using the classical phenotypical classification system, including the more sophisticated automated systems such as the VITEK 2, which often classify it as *C. famata* have been assumed to form a homogeneous taxonomic group. But this has been broken down by high resolution molecular analysis, based on genomic markers. [80] As clearly elucidated by Tavanti et al. (2005), the historical isolates of *C. parapsilosis* were actually three reproductively isolated species (*C. orthopsilosis* and *C. metapsilosis*), therefore, these new species have been formally described. As clearly elucidated by Tavanti et al. (2005), the historical isolates of *C. parapsilosis* were actually three reproductively isolated species, *C. orthopsilosis* and *C. metapsilosis,* hence the new species are formally described.[88], [89] These evolutionary differences are masked completely by automated phenotypic systems as the newly separated species have essentially the same biochemical profile for carbohydrate assimilation. To accurately map such critical high-resolution biodiversity within the fermented beverage matrix, the underlying genomic blueprint has to be extracted and interrogated.[10], [70], [87], [89], [90], [91], [92]

8. BEYOND THE NUCLEUS: MITOCHONDRIAL GENOMES AND INDUSTRIAL HYBRIDS

The modern era of molecular characterization has gone beyond conventional chromosomal markers and reveals the genome of organelles, extending insights on yeast evolution to understanding stress response and its industrial applications. The mitochondrial genome is a valuable resource in studying the adaptation of species to fermentative environments. The complete mitochondrial genome of a few species of non-Saccharomyces yeasts, including the thermotolerant, lactose-fermenting yeast *Kluyveromyces marxianus,* has been sequenced, and distinct evolutionary trends emerged concerning the gene order, the presence of introns and intergenic spacer regions when compared with the extensively studied yeast Saccharomyces cerevisiae.[93], [94] Moreover, in an industrial fermentation context, mixing of the various species of yeast is commonplace, resulting naturally in

hybrid strains that have better fermentation kinetics and robustness. These industrial hybrids have evolved through intricate processes that are revealed by genomic sequencing. The high-resolution whole genome and mitochondrial sequencing technologies are essential to characterize these complex introgression events in the genome, which are impossible without such technologies.[95], [96], [97], [98], [99], [100], [101]

9. PROTEOMIC FINGERPRINTING: MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

Molecular sequencing offers ultimate taxonomic accuracy, but is operationally limited by long turn-around times (72 to 24 hours for PCR amplification, purification, and capillary electrophoresis) and by the high cost of per-sample reagents. Over the past few years, MALDI-TOF MS has become a disruptive, very sophisticated technology that has made it possible to combine the speed of the VITEK 2 system with a diagnostic sensitivity increasingly approaching molecular sequencing.

Physical Principles of Mass Spectrometry: Instead of biochemical carbohydrate assimilation and targeted sequencing of the DNA, MALDI-TOF MS is based on high-resolution proteomic mass profiling. A small portion of a pure yeast colony is therefore smeared directly onto a special slide, made of a metal material. The biological sample is then covered with a chemical matrix solution such as most often, α-Cyano-4-hydroxycinnamic acid (CHCA)[102]. When activated inside the spectrometer chamber, the system delivers carefully-tuned pulses of UV laser light at the sample spot. The newly charged, protein backbone intact ions are then accelerated by the use of a powerful electric field through a vacuum flight tube without destroying the protein backbone.[102] Physical separation is governed by the Time-of-Flight (TOF) principle: Smaller, lighter protein ions reach the terminal detector much faster and much sooner in the vacuum than larger, heavier proteins. Data obtained is very specific often referred to as the organism's "mass spectrum" or "proteomic fingerprint" a visual display of the exact mass-to-charge (m/z) ratios of the most abundant intracellular proteins. This empirical spectrum is then subjected to extremely sophisticated algorithmic software that automatically analyzes the distribution of peaks and matches them to a large proprietary database of reference spectra, to provide a near-instantaneous identification of the specimen's taxonomic group.[13], [103], [104], [105], [106], [107]

Immunity to Phenotypic Plasticity: The main benefit of MALDI-TOF systems (e.g. the VITEK MS) over biochemical systems (e.g. the VITEK 2) is the object of the analysis. As the mass spectrometer profile is based on reaction of highly conserved abundant ribosomal proteins, the resulting spectral fingerprints are remarkably stable as they are not based on the expression of metabolic enzymes. This stability is maintained under all environmental stresses (ethanol, acid shock) that the yeast has experienced before being isolated, overcoming the ever-present problem of phenotypic plasticity in biochemical methods based on variable gene expression.[13], [104], [107], [108], [109], [110]

10. DIAGNOSTIC ECONOMICS AND TURNAROUND EFFICIENCY: MALDI-TOF VS. BIOCHEMICAL SYSTEMS

The operational and economic benefits of MALDI-TOF MS to classic phenotypic systems are remarkable and well established. The study specifically compared the proteomic MALDI-TOF (VITEK MS) with the biochemical ANC card (VITEK 2) using 421 complex, slow growing anaerobic bacteria, but the algorithms, cost analyses, and workflow principles used are applicable to yeast identifications in food and clinical laboratories[102]. From a diagnostic accuracy standpoint, the MALDI-TOF system proved to be very successful. A huge 97% of the strains that were successfully identified using both two methodologies were identified by the proteomic system in the correct genus and species, with 35 strains that were not processed by the biochemical cards due to atypical reactions or database limitations being captured.[13], [102], [104], [107], [111], [112]

10.1 MICRO-COSTING ANALYSIS AND WORKFLOW ACCELERATION

Most importantly, the reduction in turn-around time (TAT) that mass spectrometry offers is astounding. The MALDI-TOF system thus enabled the final results of positive culture to be released up to five days before the standard Vitek 2 biochemical process. The MALDI-TOF system was therefore able to deliver the results of positive culture as much as 5 days earlier than the biochemical process used in the Vitek 2.[102]. The economic ramifications are also profound per sample. An exhaustive micro-costing analysis by Tsukimoto and Rossi (2018) determined the precise input direct costs of operation for both competing methodologies.

Table 2: Comparative direct operational cost breakdown per isolate identification, synthesizing the micro-costing analysis by Tsukimoto & Rossi (2018). [102]

Consumable Input Category	VITEK 2 (Biochemical) Cost (BRL$)	MALDI-TOF (VITEK MS) Cost (BRL$)
Primary Test Substrate	17.30 (ANC Card)	1.40 (MALDI Slide Spot)
Chemical Matrix	N/A	0.04 (CHCA Matrix, 1 µL)
Application Tool	0.30 (Disposable Handle + Swab)	0.02 (Disposable Stick)
Auxiliary Media/Slides	1.97 (Agar Plate + Gram Slide)	N/A
Total Direct Cost per ID	**Approx. BRL$ 20.00**	**BRL$ 1.50**

The empirical evidence confirms that the biochemical cost of a single identification can be reduced by using the MALDI-TOF platform by around 13% and the direct cost is even lower by using the MALDI-TOF platform, which comes to BRL$ 7,786 for the period of the specific study that evaluated 421 samples, confirming the economic shift. Other more extensive institutional evaluations have corroborated this economic shift, noting reductions in diagnostic cost at the level of 89.3% upon the implementation of mass spectrometry[102]. However, it should be stressed that the cost of the initial capital investment for MALDI-TOF mass spectrometer is very high, ranging from BRL$ 600,000 to BRL$ 800,000, or hundreds of thousands of US dollars. Automated phenotypic readers, on the other hand, come with a much lower initial investment. However, in high throughput laboratories where complex environmental, clinical and food samples are analyzed on a regular basis, the

tremendous decrease in per-sample consumable costs coupled with the unparalleled diagnostic speed provides a quick and compelling Return on investment (ROI).[102], [104], [107], [113], [114]

Table 3. Comparative analysis of methods used for yeast identification in traditional fermented beverages. **[10], [13], [14], [56], [69–71], [80], [82], [84–87], [102–110], [113], [116].**

Parameter	Classical Biochemical Tests	VITEK 2 Compact	RapID Yeast Plus	MALDI-TOF MS	ITS/D1/D2 Sequencing
Turnaround time	7–14 days	15–18 h	4–24 h	Minutes	24–72 h
Species-level accuracy	Moderate (70–85%)	High (90–95%)	High (94–96%)	High (>95%)	Very high (>99%)
Strain-level resolution	No	No	No	Limited	Limited
Cryptic species discrimination	Poor	Poor	Poor	Moderate	Excellent
Effect of phenotypic plasticity	High	High	High	Minimal	None
Database dependence	Low	High	High	High	Moderate (GenBank/UNITE)
Throughput	Low	Moderate	Moderate	Very high	Moderate
Per-sample cost	Low	Moderate	Moderate	Low	High
Initial equipment cost	Low	Moderate	Low	Very high	Moderate
Need for pure culture	Yes	Yes	Yes	Yes	Usually yes
Detection of unculturable microorganisms	No	No	No	No	Possible through metagenomics
Suitability for routine diagnostics	Moderate	High	High	Excellent	Moderate
Suitability for food microbiology	Good	Good	Moderate	Excellent	Excellent
Suitability for biodiversity studies	Poor	Poor	Poor	Good	Excellent

Recommended application	Physiological characterization	Routine identification	Rapid clinical identification	High-throughput screening	Definitive taxonomic identification

11. FUTURE PERSPECTIVES AND POLYPHASIC TAXONOMY INTEGRATION

The traditional fermented beverages are composed of a wide variety of yeast species, and none single technology is enough to accurately characterise them. While classical phenotypic testing is useful for ethanol tolerance, killer activity and flocculation traits of industrial strains, MALDI-TOF MS would be a valuable tool for routine identification which is quickly and economically achieved. Despite this MALDI-TOF MS and molecular methods have their own limitations in the size of their reference databases. Hence, MALDI-TOF MS is needed for high throughput screening of starter cultures and further identification by ITS and D1/D2 sequencing, biochemical assays to characterize their functional properties of potential starter cultures [9, 35, 53, 84, 87, 106, 116, 117].

Next-Generation Sequencing and Metagenomics: Culture-independent analysis of microbial communities in fermented beverages has been possible thanks to recent developments in next-generation sequencing (NGS). Amplicon sequencing and metagenomics offer a holistic view of microbial diversity, succession and relationships, even those of microorganisms that cannot be cultured.[115], [116]

Whole genome sequencing and pangenomics: Whole genome sequencing (WGS) can offer strain level resolution and is able to unravel genetic traits related to stress tolerance, fermentation performance and aroma production. Pangenomic analyses also include the identification of core and accessory genes, which helps identify novel starter cultures.[117], [118], [119]

Multi-Omics and Artificial Intelligence: The multi-omics integrated approaches are used to gain the systemic understanding of fermentation processes such as transcriptomics, proteomics, and metabolomics. At the same time, artificial intelligence and machine learning is being used to analyse MALDI-TOF spectra and genome data sets to enhance microbial identification and precision fermentation.[106], [120]

Future Outlook: The use of a polyphasic approach, which involves both phenotypic methods and MALDI-TOF MS, targeted sequencing, whole genome analysis and multi-omics approaches, will be the basis for future yeast characterization, enabling accurate taxonomic identification and functional characterization of industrially important yeast strains.[10], [14], [87]

12. CONCLUSIONS

Traditional fermented beverages are hyper-complex and dynamic biochemical reactors, with various interacting communities of Saccharomyces and non-Saccharomyces yeasts. Accurate delineation of these ecosystems is essential to efficiently scale up artisanal beverages, control against spoilage by resilient microorganisms such as *Zygosaccharomyces bailii* and utilize new health benefits of probiotics. This comprehensive comparative study clearly shows that classical or automated phenotypic profiling systems are vulnerable from taxonomic point of view in the context of food isolates from environmental stress. Systems such as Vitek 2 have great promise for rapid kinetic cycle, targeted, clinical application, but are hampered by mechanical substrate limitations which make it impossible to break down cryptic species complexes, and by the very sensitivity of the system to

environmentally induced phenotypic plasticity. Molecular genotyping, in particular using the highly variable ITS and the ~600 bp D1/D2 ribosomal domains, remains the definitive "gold standard" for establishing genuine evolutionary relationships and resolving deep-seated taxonomic ambiguities, including the separation of *Candida orthopsilosis* from the *C. parapsilosis* complex. Because of its speed, lower cost per sample and increased accuracy, MALDI-TOF MS has been gradually replacing the traditional biochemical identification systems in many routine laboratories. However, biochemical tools remain secondary to the phenotypic ones for use in a polyphasic taxonomic approach. Finally, the prospects of yeast identification in traditional fermented drinks are not a single high-tech solution, but a smart and hierarchical application of the polyphasic taxonomy. The discipline can combine the rapidity of proteomic mass screening of the environment with the high-resolution power of genomic sequencing for definitive taxonomic resolution, and the precision of biochemical profiling for targeted analysis of enzymes and their function, to obtain complete, actionable mapping of the global fermentation microbiome.

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