



The diversity of microflora in cider production and methods for identifying *Saccharomyces* and non-*Saccharomyces* yeasts: A review

Anastasia A. Shirshova*^{ORCID}, Natalia M. Ageeva^{ORCID}, Anton A. Khrapov^{ORCID},
Ekaterina A. Al-Nakib^{ORCID}, Julia N. Chernyavskaya^{ORCID}, Elena V. Lobodina^{ORCID}, Ekaterina V. Globa^{ORCID}

North Caucasian Federal Scientific Center of Horticulture, Viticulture, Wine-making^{ORCID}, Krasnodar, Russia

e-mail: anastasiya_1987@inbox.ru

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Abstract:

Recently, yeasts of the genus *Saccharomyces* have been increasingly used to make ciders. However, spontaneous microflora has been traditionally used to bring out unique sensory characteristics determined by the terroir. Although the use of *Saccharomyces* has been extensively studied, there has been little research into use of natural microflora (bacteria and fungi, including yeasts) of apple must in Russian cider production. Therefore, we aimed to analyze the diversity, role, and metabolic products of microorganisms involved in various biochemical processes during cider production, as well as to review the methods for identifying *Saccharomyces* and non-*Saccharomyces* yeasts.

We reviewed scientific papers published in 2001–2026 and studying microbial communities involved in cider production, as well as methods for their isolation and identification. The publications were sourced from the bibliographic databases of Scopus, PubMed, Elsevier, ResearchGate, Google Scholar, and eLIBRARY.RU. The data were analyzed, summarized, and systematized. Ciders with the most complex and harmonious sensory characteristics are produced by mixed fermentation using non-*Saccharomyces* and *Saccharomyces* yeasts. The most commonly methods used for microorganism identification are polymerase chain reaction and high-throughput sequencing. The diversity of natural microflora depends on many factors, including the geographic location (terroir) of cider production.

Further research is needed to identify which *Saccharomyces* species and non-*Saccharomyces* genera predominate in the natural microflora of apple fruits used for cider production in Russia. Also, we need to improve the existing methods, or develop new techniques, for identifying microflora and assessing its effect on the quality of the beverage as early as during fermentation.

Keywords: Apple fruits, cider, microorganisms, *Saccharomyces*, non-*Saccharomyces*, lactic acid bacteria, acetic acid bacteria, mold fungi, identification methods

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INTRODUCTION

This article is a review of literature on cider production. In particular, we studied the diversity of microorganisms involved in related biochemical processes, as well as their role and metabolic products. We also analyzed methods for identifying *Saccharomyces* and non-*Saccharomyces* yeasts.

Cider is a low-alcohol beverage obtained by fermenting fresh apple must. Fermentation converts apple must into cider under the action of various yeasts and bacteria, including malolactic bacteria, during numerous

biochemical processes [1, 2]. The natural microflora of cider is a community of yeasts and bacteria of various genera, species, and strains. Of them, yeasts of the genus *Saccharomyces* are mainly responsible for alcoholic fermentation, whereby the yeast enzymatic systems convert sugars into ethyl alcohol and carbon dioxide, the primary fermentation products [3]. The secondary fermentation products, which come in significantly lower concentrations, are responsible for certain sensory characteristics of cider. They include organic acids, amino acids, aromatic volatile substances, B vitamins, rutin, and other components [4].

Apple must can be fermented by spontaneous (natural) or controlled methods. Spontaneous fermentation is enabled by the fruit's natural microflora, which largely depends on the apple variety and place of origin [5, 6]. Controlled fermentation, in contrast, involves inactivating the natural microflora with sulfur preparations and adding pure yeast cultures, primarily *Saccharomyces*. However, these two approaches can be combined or mixed. Mixed fermentation starts with the natural microflora, mainly non-*Saccharomyces* yeasts, converting sugars into alcohol. When ethyl alcohol reaches 2.0–4.0% ABV (alcohol by volume), the natural microflora dies and *Saccharomyces* yeasts are added to continue the process. It should be noted that fermentation is characterized by the accumulation of various metabolites, which can significantly alter the quality of cider, as well as its aroma and flavor [7, 8]. Therefore, understanding the process and managing yeast and bacterial communities can reduce or prevent potential risks in cider production.

There has been far less research into cider production than wine making, especially research on the role of the fruit's natural microflora in fermentation. Modern wine makers are taking a lot of interest in learning how the grape's natural microflora can enhance the wine's sensory properties, as well as isolating pure yeast cultures from the grape's surface. However, with all the similarities between cider and wine in terms of their microbiome and fermentation process (both alcoholic and malolactic), apples and grapes have different compositions and properties, with specific production and environmental parameters required in each case. Therefore, despite extensive research into wine making, research into cider production remains highly relevant [9].

Krasnodar Krai (south-west of Russia) boasts abundant apple orchards that yield rich harvests not only for fresh consumption but also for juice and cider production. However, there has been little research into the microbiological status of apple fruits to isolate microflora for cider production. According to literature, knowing what microbial species are present in the microflora, as well as their quantity and activity, is vital for producing cider of consistent quality. This involves identifying and quantifying microbial populations, as well as studying their metabolic networks, on the surface of harvested apple fruits. Such research can elucidate what mech-

anisms regulate the formation of beneficial microbial communities and how they can be manipulated to produce high-quality ciders. Ultimately, this may help Russian producers replace imported active dry yeasts with domestically produced substitutes. In this review, we aimed to analyze and systematize the findings of studies on the diversity of microorganisms used in cider production, including *Saccharomyces* and non-*Saccharomyces* yeasts, as well as methods for their identification.

STUDY OBJECTS AND METHODS

We studied relevant scientific articles published in 2001–2026 on the properties and metabolic products of apple surface and cider microflora, as well as methods for identifying *Saccharomyces* and non-*Saccharomyces* yeasts used in cider production.

The papers were sourced from the databases of Scopus, PubMed, Elsevier Science, ResearchGate, Google Scholar, and eLIBRARY.RU using keywords and their combinations.

RESULTS AND DISCUSSION

The natural apple surface microflora can be divided into four groups: yeasts (*Saccharomyces* and non-*Saccharomyces*), lactic acid bacteria, acetic acid bacteria, and mold fungi (Fig. 1).

The main anaerobic microorganisms involved in the fermentation of apple must are 1) *Saccharomyces* yeasts (*Saccharomyces cerevisiae*, *Saccharomyces bayanus* Sacc., 1895, *Saccharomyces uvarum* Beij., etc.) and non-*Saccharomyces* (*Brettanomyces*, *Metschnikowia pulcherrima* Pitt & M.W. Mill. (1968), *Hanseniaspora valbyensis* Klöcker (1912), (*Kloeckera apiculata* (Reess) Janke (1923)), *Hanseniaspora uvarum* (Niehaus) Shehata, Mrak & Phaff (1956), *Hanseniaspora vineae* Van der Walt & Tschuschner (1957), *Hansenula anomala* (E.C. Hansen) Syd. & P. Syd. 1919, *Torulopsis*, *Torulaspora delbrueckii* (Lindner 1895) Lindner 1904, *Kluyveromyces thermotolerans* (Philippov 1932) Yarrow 1972, *Hanseniaspora osmophila* (Niehaus) Phaff, M.W. Mill. & Shifrine (1956), etc.); 2) lactic acid bacteria of the family *Lactobacillaceae* (*Oenococcus oeni*, *Pediococcus*, *Lactobacillus*, *Leuconostoc*, etc.); 3) acetic acid bacteria of the family *Acetobacteraceae* (*Acetobacter*, *Gluconobacter*, etc.); and

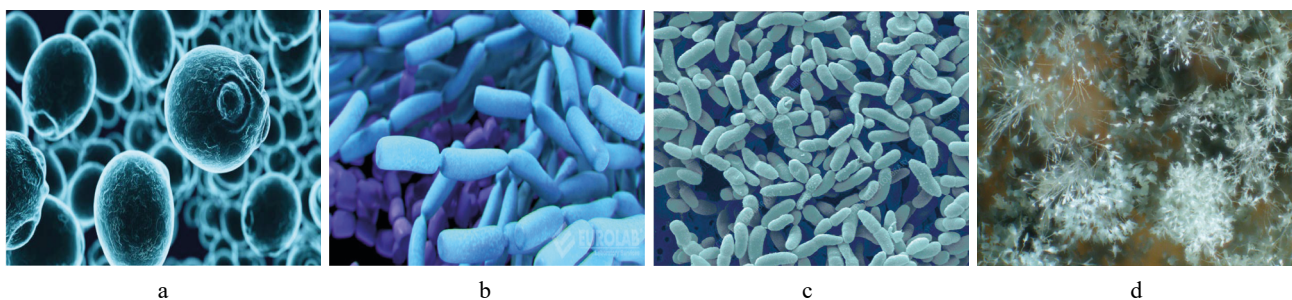


Figure 1 Microorganisms involved in fermentation of apple must: a – yeasts (*Saccharomyces*); b – lactic acid bacteria; c – acetic acid bacteria; and d – mold fungi (sourced from ru.wikipedia.org, magnified $\times 1000$ (a, b, c) and $\times 400$ (d))

4) mold fungi (*Aspergillus*, *Penicillium expansum* Link 1809, *Botrytis cinerea* Pers. (1794), *Alternaria alternata* (Fr.) Keissl., *Diplodia seriata* De Not. (1845), *Monilinia fructigena* (Pers.) Honey, etc.) (Table 1).

Controlled fermentation involves the use of a pure culture of *Saccharomyces* yeast in the form of a yeast starter or an active dry yeast preparation. This fermentation technique allows for the production of cider with desired sensory characteristics and consistent quality, minimizing the risk of faults [10, 11]. It is therefore preferred by large-scale cider producers.

Saccharomyces yeasts are unicellular fungi with a diploid genome consisting of 16 pairs of chromosomes. Its species can reproduce by budding, producing a copy of a gene and an organelle and dividing into two parts, or by sporulation. For beverage production, yeasts that do not form spores or form them in small quantities are selected to ensure the stability of fermentation.

Saccharomyces species include *S. cerevisiae*, *S. bayanus*, *S. uvarum*, *Saccharomyces cariocanus* G.I. Naumov, S.A. James, E.S. Naumova, E.J. Louis & I.N. Roberts (2000), *Saccharomyces eubayanus* J.P. Samp., Libkind,

Table 1 Microorganisms used in cider production

Genus/Species	Characteristic	Sensory properties of cider	Reference
<i>Saccharomyces cerevisiae</i>	Promote the formation of ethyl esters (ethyl hexanoate, ethyl decanoate, ethyl octanoate), octanoic and decanoic acids	Clean, bright aroma and a balanced rich taste	[11, 13]
<i>Saccharomyces bayanus</i> Sacc., 1895	Provide a high fermentation rate (up to 7 days at 10–12°C), with a residual sugar mass of 0.6–4.0 g/L, fairly high concentrations of glycerol, succinic acid, and volatile compounds such as 2-phenylethanol and volatile thiols	Pronounced fruity-floral notes in aroma and taste	[4, 14]
<i>Saccharomyces uvarum</i> Beij.	Tolerant of low temperatures (suitable for ice cider production); able to produce high concentrations of glycerol, 2-phenylethanol, and 2-phenylethyl acetate and low concentrations of acetic acid; and metabolize sorbitol	Complex, bright aroma; rich, rounded taste, with original “terroir” sensory characteristics	[15, 22–25]
<i>Hanseniaspora</i> (<i>Hanseniaspora vineae</i> Van der Walt & Tscheuschner (1957))/Kloeckera	Produce significant quantities of esters (2-phenylethyl acetate and ethyl acetate), higher alcohols, and volatile phenols. Used for co- or sequential fermentation to achieve a more complex cider aroma	Aroma with fruity (apple, pear, pineapple) and floral (rose) notes and hints of honey; soft taste	[19, 26–29]
<i>Candida</i> (<i>Candida mycoderma</i> (Reess) Lodder & Kreger-van Rij (1952), <i>Mycoderma vini</i> J.N. Vallot ex Desm. (1823), <i>Candida zemplinina</i> Sipiczki (2003))	Form a thin white film on the surface of cider (flor) due to oxygen exposure; convert ethyl alcohol into ethyl acetate as biomass accumulates; and require strict control. Co-fermentation with <i>Saccharomyces cerevisiae</i> and <i>Candida zemplinina</i> Sipiczki (2003) reduces acetic acid content by 0.3 g/L while maintaining high ethyl alcohol and glycerol levels	Sherry-like notes in aroma and taste (in controlled fermentation); dull appearance and off-odors such as the smell of solvent or musty flavor (in uncontrolled fermentation)	[30, 31]
<i>Brettanomyces</i> (<i>Brettanomyces bruxellensis</i> Kuff. & van Laer (1954), <i>Brettanomyces anomalus</i> Custers (1940))	Reduce the contents of ethyl alcohol and esters and contribute to high concentrations of fatty acids and their ethyl esters, volatile phenols (acetate esters, 4-ethylcatechol, 4-ethylphenol, and 4-ethylguaiacol) from caffeic, <i>p</i> -coumaric and ferulic acids	Notes of leather, barnyard, horse saddle, smoke, adhesive tape and other medicinal and metallic shades, as well as pleasant fruity (apple) and spicy (clove, curry, etc.) notes in aroma	[17, 32–37]
<i>Metschnikowia pulcherrima</i> Pitt & M.W. Mill. (1968), <i>Metschnikowia koreensis</i> S.G. Hong, Chun, Oh & Bae (2001), <i>Metschnikowia reukauffii</i> Pitt & M.W. Mill. (1968)	Suitable for low-alcohol ciders; produce high concentrations of glycerol, acetate esters, and higher alcohols, and low concentrations of acetic acid. Used to regulate acidity by reducing malic acid and increasing lactic acid	Improved color, complex aroma (floral, herbal, fresh fruit notes) and rounded flavor	[38–40]
<i>Torulaspora delbrueckii</i> (Lindner 1895) Lindner 1904	Synthesize high concentrations of polyols – polyhydric alcohols (glycerol, arabitol, ribitol, sorbitol, mannitol, and xylitol) and esters – that protect yeast cells from osmotic stress. Therefore, suitable for fermenting late-harvest apples and producing ice ciders. Exhibit a high sedimentation rate, resulting in higher color intensity and lower concentrations of total and monomeric anthocyanins	Complex flavor with fruity notes	[1, 41]

Continuation of Table 1

Genus/Species	Characteristic	Sensory properties of cider	Reference
<i>Kluyveromyces marxianus</i> (E.C. Hansen) Van der Walt (1971)	Produce esters – ethyl acetate, hexyl acetate, propyl acetate, 2-phenylethanol, and acetate	Complex flavor with fruity notes	[13]
<i>Pichia kluyveri</i> Bedford ex Kudryavtsev	Produce acetate esters (3-methylbutyl acetate)	Tropical (banana) and fruity notes in aroma	[42–44]
<i>Pichia barkeri</i> Phaff, Starmer, Tredick & V. Aberdeen (1987), <i>Pichia kudriavzevii</i> Boidin, Pignal & Besson, 1965	Reduce concentrations of titratable acids and acetic acid, increase glycerin concentrations	Complex, rounded flavor	
<i>Schizosaccharomyces pombe</i> Lindner, 1893	Used to adjust concentrations of titratable acids. Deacidify L-malic acid to ethyl alcohol; produce large quantities of volatile fatty acids, such as acetic acid, and low concentrations of higher alcohols	Softer, rounded flavor	[18, 45, 46]
<i>Zygosaccharomyces hancea</i>	Promote the formation of volatile fatty acids, such as acetic acid, and low concentrations of higher alcohols	Enhanced aroma and flavor with floral, sweet, and woody tones	[47]
<i>Rhodotorula mucilaginosa</i> (A. Jörg.) F.C. Harrison, 1928	Reduce concentrations of titratable acids and increase contents of esters (isoamyl acetate, phenylethyl acetate, ethyl lactate) and higher alcohols (1-butanol)	More transparent and brighter color, more intense aroma with fruity and floral notes; complex taste	[48]
<i>Lachancea thermotolerans</i> (Filippov 1932) Kurtzman 2003	Promote lactic acid production (2.0–9.0 g/L) from glucose, significantly increasing concentrations of titratable acids and lowering the pH of cider. Produce aromatic compounds (ethyl lactate and other esters, 2-phenylethanol, linolol, etc.) and glycerol, as well as some terpenes	Aroma with fruity and floral notes; balanced acidity	[49–51]
Family <i>Lactobacillaceae</i> : <i>Oenococcus oeni</i> , <i>Pediococcus</i> , <i>Lactobacillus</i> , <i>Leuconostoc</i> , etc.	<i>Oenococcus oeni</i> function at low pH, are resistant to high concentrations of titratable acids and high ethyl alcohol levels, produce esters, and convert malic acid into lactic acid. <i>Pediococcus</i> are lactic acid bacteria that slow down the growth of yeast, destroy the taste and aroma, cause cider spoilage, increase concentrations of titratable acids and diacetyl, and form viscous mucus (“threads”). <i>Leuconostoc</i> and <i>Lactobacillus</i> promote the formation of volatile compounds, diacetyl, and acetoin; exhibit cinnamoyl esterase activity, which reduces concentrations of organic acids in ciders	<i>Oenococcus oeni</i> – creamy and cheesy, fruity and floral notes in aroma; soft and rounded flavor. <i>Pediococcus</i> – oily and rancid smell, viscous mucus. <i>Leuconostoc</i> and <i>Lactobacillus</i> – complex aroma with pleasant creamy, buttery, and nutty notes	[9, 18, 23, 34, 52–58]
Family <i>Acetobacteraceae</i> (<i>Acetobacter</i> and <i>Gluconobacter</i>)	Oxidize alcohol to acetic acid, followed by further oxidation of acetic acid to carbon dioxide and water, while synthesizing certain organic acids and volatile aromatic compounds (esters, ketones, and aldehydes). Acetic acid fermentation results in lower ethyl alcohol levels and higher concentrations of volatile and titratable acids	Notes of acetic acid in aroma and taste	[59, 60]
<i>Aspergillus</i> , <i>Penicillium expansum</i> Link 1809, <i>Botrytis cinerea</i> , <i>Alternaria alternata</i> (Fr.) Keissl., <i>Diplodia seriata</i> De Not. (1845), <i>Monilinia fructigena</i> (Pers.) Honey	When apple fruits infected with <i>Penicillium</i> and <i>Aspergillus</i> are processed into cider, a toxin patulin is formed, which poses a serious threat to human health. <i>Botrytis cinerea</i> and <i>Botrytis cinerea</i> Fr. are gray mold pathogens, harmful parasitic micromycetes that can damage yields and destroy stored apple fruits. <i>Alternaria alternata</i> (Fr.) Keissl., <i>Diplodia seriata</i> De Not. (1845), and <i>Monilinia fructigena</i> (Pers.) Honey are apple fungi that significantly degrade the quality of cider, altering its chemical composition and sensory properties. They increase concentrations of volatile acids and decrease concentrations of higher alcohols and esters	Deteriorated aroma due to mold tones and the loss of fruity notes in aroma and flavor, bitter taste	[9, 57–64]

Hittinger, P. Gonç., E. Valério, C. Gonç., Dover & M. Johnst, *Saccharomyces kudriavzevii* G.I. Naumov, S.A. James, E.S. Naumova, E.J. Louis & I.N. Roberts (2000), *Saccharomyces mikatae* Naumov et al., 2000, *Saccharomyces paradoxus* Bachinskaya, 1914, *Saccharomyces pastorianus* Reess ex E.C. Hansen, and others. Each of the species has a variety of strains, with *S. cerevisiae* strains numbering thousands.

S. cerevisiae yeast is traditionally used in baking, brewing, winemaking, cider and kvass production, as well as for medicinal and preventative purposes as a source of B vitamins [12]. Cider producers worldwide use this yeast species due to its high fermentation activity. During fermentation, it synthesizes a number of major compounds, including amino acids, organic acids, vitamins, and volatile compounds (higher alcohols and volatile esters). These compounds contribute to the characteristic aroma and flavor of cider. However, the qualitative and quantitative composition of volatile compounds depends on the yeast strain, since different *S. cerevisiae* strains synthesize different groups of volatile compounds in varying concentrations. Therefore, the choice of a particular *S. cerevisiae* strain is crucial for the production of high-quality cider [7, 13].

S. bayanus yeasts differ from *S. cerevisiae* yeasts in that they produce lower concentrations of acetic acid and ethyl alcohol, but higher concentrations of glycerol and succinic acid. Furthermore, *S. bayanus* yeasts synthesize malic acid without subsequent transformation, as well as volatile compounds such as 2-phenylethanol and volatile thiols [14–16]. These compounds contribute to the pronounced fruity-floral notes in the aroma and flavor of cider. Finally, this yeast species ensures high rates of complete sugar fermentation at low temperatures (10–12°C).

S. uvarum yeasts are also tolerant of low temperatures, as well as high ethyl alcohol levels (good for ice cider) and sulfites. Furthermore, they metabolize sorbitol and produce high concentrations of glycerol, 2-phenylethanol, and 2-phenylethyl acetate and low concentrations of acetic acid.

Spontaneous fermentation of fresh apple must occurs under the influence of natural microflora found on the fruit's surface and flesh, as well as in the processing equipment after the fruit skin is broken. Smaller producers often utilize local apple varieties and natural microflora, including wild yeast, to bring out the “terroir” characteristics in cider. A good example is French cider produced primarily in the northwestern regions of France (Brittany and Normandy). Its distinctive characteristics distinguish it from ciders from other countries [17].

Scientists from various countries (China, Poland, Serbia, and Portugal) are taking considerable interest in microorganisms capable of spontaneous fermentation [18].

The main non-*Saccharomyces* yeasts found on the surface of apple fruits are *M. pulcherrima*, *H. valbyensis* (*K. apiculata*), *H. uvarum*, *H. anomala*, *H. osmophila*, *H. vineae* *K. thermotolerans*, *Torulopsis*, *T. delbrueckii*, *Brettanomyces*, *O. oeni*, and others [19]. According to Bartuv et al. [20] and Wei et al. [21], the surface of fresh

apple fruits is an important source of autochthonous yeasts, which can initiate spontaneous fermentation or serve as starter cultures in mixed fermentation.

According Flores et al. [65], Tyakht et al. [66], and Villar et al. [67], natural fermentation with spontaneous microflora results in cider having more complex sensory characteristics due to the formation of secondary fermentation products. The diversity of microbial communities and the rate of sugar reduction during spontaneous fermentation vary significantly depending on apple variety and place of origin. These variables directly correlate with the qualitative and quantitative composition of volatile compounds in cider. Therefore, the use of local natural microflora to enrich the flavor of cider and give it a unique character, is always determined by a specific terroir, i.e., the place where apples are grown and processed [42]. However, natural microflora is difficult to control and its use has a number of disadvantages, including higher contents of volatile acids, biogenic amines, hydrogen sulfide, and other undesirable components in cider [68]. Moreover, spontaneous fermentation may suddenly stop due to a gradual decrease in non-*Saccharomyces* yeasts caused by their low tolerance to ethyl alcohol, whose content increases during fermentation.

Mixed fermentation is a process using several types of microorganisms (yeasts and/or bacteria) simultaneously or sequentially to produce beverages with more complex aromas and flavors.

Recent decades have seen increased interest in using non-*Saccharomyces* (*Kloeckera*, *Saccharomycodes*, *Schizosaccharomyces*, *Hansenula*, *Candida*, *Pichia*, *Torulopsis*, etc.) with *S. cerevisiae* and/or *S. bayanus* in mixed or sequential fermentation. Non-*Saccharomyces* produce esters and volatile acids in significant concentrations, which has a beneficial effect on the taste and aroma of cider in controlled fermentation [69]. However, these yeasts are not alcohol-resistant and they die when ethyl alcohol reaches 2.0–4.0% in the fermenting must. When it happens, *Saccharomyces* yeasts take over and complete the fermentation process, providing cider with the required alcohol content and characteristic fermentation notes. Ciders produced by combining natural microflora and pure yeast cultures have a more complex aroma and flavor with fruity, floral, herbal, and other notes [70].

Hanseniaspora/Kloeckera is a non-*Saccharomyces* species isolated from the spontaneous microflora of apple fruits. According to Wyk et al. [71], *Hanseniaspora apiculata* Kudryavtsev can stop fermentation, especially at low concentrations of nitrogen compounds in the must, by suppressing the reproductive function of wine yeast cells. However, a study by Pietrowski et al. [28] showed that some strains of the *H. vineae* yeast can enhance the aroma of cider by producing significant quantities of esters, volatile acids, phenols, and higher alcohols, primarily 2-phenylethyl acetate and ethyl acetate. *Hanseniaspora* yeasts are also combined with *Saccharomyces* in order to obtain a softer taste and a more complex aroma with fruity (apple, pear, pineapple) and floral (rose, honey) notes [29].

Candida. To obtain a stable and complex flavor, cider producers sometimes resort to mixed fermentation, combining *Saccharomyces* and *Candida* [61]. For example, co-inoculation of *S. cerevisiae* and *Candida zemplinina* Sipiczki (2003) reduced the content of acetic acid, while maintaining high concentrations of ethyl alcohol and glycerol [31].

Yeasts of the genus *Brettanomyces*, also known as wild or contaminant yeasts, are a serious cause of microbial spoilage in the production of fermented beverages. *Brettanomyces* cells are oval or round in shape, often with pointed ends, and they are much smaller than those of *S. cerevisiae*. Some strains have significantly elongated cells that are often fused in groups of two or more. In a favorable nutrient medium, most strains exhibit a tendency to form pseudomycelium [72].

Brettanomyces yeasts accumulate significant amounts of C8–C12 fatty acids. Esterification reactions can lead to the formation of ethyl esters, including fatty acid esters, which are associated with certain aromas. For example, ethyl acetate contributes to a fruity aroma that can turn into a solvent-like odor with increasing concentrations. Ethyl lactate contributes to fruity and oily aromas, while phenylacetate can give cider floral (rose) notes [31].

However, the metabolic products of *Brettanomyces* do not always have a positive effect on the quality of ciders. The metabolic activity of *Brettanomyces* enabled by acetate esterase, decarboxylase, and vinylphenol reductase produces volatile phenols (4-ethylcatechol and 4-ethylphenol from caffeic and *p*-coumaric acids, respectively). These phenols may cause irreversible faults in the beverage's flavor and aroma known as the "Brett character," which is often described as a horse, leather, phenolic, medicinal, metallic, or spicy character. For example, 4-ethylphenol detected at 0.7 mg/L in Spanish cider caused a pronounced "Brett character" with notes of leather, barnyard, horse saddle, smoked meats, and adhesive tape [34]. In French ciders, 4-ethylcatechol and 4-ethylphenol detected at 12 and 3 mg/L, respectively, gave the product a phenolic flavor [69]. In addition, under aerobic conditions, *Brettanomyces* yeasts exhibit a high capacity to synthesize acetic acid.

The use of yeasts of the genus *Metschnikowia* can expand the diversity of cider's sensory characteristics. For example, the species *M. pulcherrima* produce hydrolytic enzymes that can affect both the sensory and the technological properties of cider. The microflora of ciders was found to contain *Metschnikowia koreensis* S.G. Hong, Chun, Oh & Bae (2001), *Metschnikowia reukaufii* Pitt & M.W. Mill. (1968), and *M. pulcherrima* species [40].

M. pulcherrima yeasts are used in mixed fermentations to produce low-alcohol ciders. They produce high concentrations of glycerol, acetate esters, and higher alcohols, as well as low concentrations of acetic acid. *M. pulcherrima* yeasts are renowned for their high enzymatic activity, particularly in producing terpenes (linalool, geraniol, citral, citronellol, nerol, etc.), which are contained in apple fruits and must as odorless gly-

cosides. Under the action of α -arabinofuranosidase and β -glucosidase, terpenes become "aromatically active," contributing to the typical character, aroma, and flavor of ciders [38]. Co-fermentation using *S. cerevisiae* and *M. pulcherrima* provides ciders with a more complex and vibrant aroma by producing a diversity of volatile components, such as phenylethyl alcohol, 3-methyl-1-butanol, ethyl octanoate, and decanoic acid [40].

T. delbrueckii yeasts synthesize higher alcohols and esters (butyrates, propionates, acetates), which contribute to more complex fruity and floral notes in the aroma and flavor of cider. They also promote increased glycerol production, contributing to a softer and rounded flavor. *T. delbrueckii* yeasts produce lower concentrations of volatile acids, expressed as acetic acid, than many *Saccharomyces* strains. Furthermore, they exhibit regular (uniform) fermentation kinetics, particularly in a carbon dioxide atmosphere. Their excellent foaming and effervescent properties make them suitable for the production of sparkling ciders and wines [73, 74].

In addition, mixed fermentation using *T. delbrueckii* and *S. cerevisiae* promotes increased contents of volatile aroma compounds, such as 2-phenylethanol, benzaldehyde, phenylethyl acetate, octanoic and hexanoic acids. These compounds contribute to characteristic apple, floral, and honey notes in cider's aroma [75].

Kluyveromyces marxianus (E.C. Hansen) Van der Walt (1971) yeasts promote the synthesis of esters (ethyl acetate, hexyl acetate, propyl acetate, 2-phenylethanol) and acetic acid [13]. They also increase the rate of glycolysis, which is a breakdown of one glucose molecule into two molecules of pyruvic acid (pyruvate) in the cell cytoplasm. This process is accompanied by a rapid release of energy, producing 2 ATP molecules, 2 NADH molecules, and ethyl alcohol. Limited access to oxygen decreases the metabolic flow of acetate into acetyl-CoA and then increases the synthesis of ethyl acetate and the corresponding esters in the tricarboxylic acid cycle, resulting in enhanced aroma and flavor [76]. Ester synthesis is enabled by a large group of enzymes characteristic of *K. marxianus*, most importantly alcohol acetyltransferases and esterases. Therefore, it is generally accepted that *K. marxianus* and *S. cerevisiae* have similar genetic characteristics [77]. In addition, the physiological properties of *K. marxianus* yeasts make them suitable for biotechnological production of fermented products. These eukaryotic microorganisms have the highest growth rate and a wide range of thermotolerance compared to other yeasts [78, 79].

Pichia yeasts, when co-fermented with *S. cerevisiae*, promote acetate esters (3-methylbutyl acetate, ethyl decanoate, and ethyl butyrate), which contribute to the tropical (banana) and fruity aromas in cider [44]. They are used to reduce concentrations of titratable acids and acetic acid and to increase the content of glycerol, thereby adding complexity and roundness to the cider flavor [8]. However, in uncontrolled fermentation, *Pichia alcoholophila* Klöcker yeasts can oxidize sugars, alcohols, and organic acids (malic acid). Moreover, they can

actively develop and form a film on the surface of cider. In fermented apple must, *P. alcoholophila* yeasts promote the accumulation of acetaldehyde and contribute to oxidized notes in the beverage.

Yeasts of the genus *Schizosaccharomyces pombe* Lindner, 1893 are widely used in winemaking for biological acid reduction (deacidification) in order to impart softness and roundness to an overly fresh flavor [80]. *S. pombe* yeasts can rapidly deacidify malic acid, converting it into carbon dioxide and pyruvic acid, which is then transformed into ethyl alcohol. The yeasts are also used to reduce the concentration of malic acid in cider, resulting in lower concentrations of titratable acids compared to cider produced with only *S. cerevisiae* [49]. Finally, *S. pombe* yeasts are now used in the production of other beverages due to their ability to biologically reduce acidity and form glycerol, esters, and polysaccharides, which determine the sensory characteristics of wines and ciders [81].

Yeasts of the genus *Zygosaccharomyces* are tolerant of high osmotic pressure, high concentrations of titratable acids, sulfur dioxide, and low pH. They utilize fructose to a greater extent than glucose. *Zygosaccharomyces hancea* yeasts synthesize higher levels of ethyl alcohol and volatile esters than *S. cerevisiae*. They promote volatile acids such as acetic acid and reduce concentrations of higher alcohols. In co-fermentation with *S. cerevisiae*, they improve the aroma and flavor of cider by producing floral, sweet, and woody notes [82].

Yeasts of the genus *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison, 1928 are non-*Saccharomyces* with low fermentation activity. Isolated from the surface of apple and grape fruits, they are typically used in the first stage of fermentation of fruit wines and ciders.

The co-fermentation of apple must by *R. mucilaginosa* and *S. cerevisiae* results in significantly higher levels of 1-butanol and ethyl lactate in cider compared to using only *S. cerevisiae* [40].

Yeasts of the genus *Lachancea thermotolerans* (Filippov 1932) Kurtzman 2003 produce high concentrations of lactic acid (2.0–9.0 g/L) from glucose, which significantly increases concentrations of titratable acids and lowers the pH of cider. Furthermore, they promote aromatic compounds (esters, 2-phenylethanol, linoleic acid, etc.) and glycerol, as well as some terpenes [51].

Malolactic fermentation is an important stage in cider production that improves the sensory characteristics of the beverage. Malolactic fermentation is induced by pure bacterial cultures for better control. This is a common practice for biological acidification in winemaking. The same malolactic bacteria are most often used in cider production [57].

Malolactic fermentation can occur spontaneously – simultaneously with, or after, alcoholic fermentation – under the action of malolactic bacteria such as *Oenococcus*, *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, which are part of the natural microflora.

The genus *Oenococcus* is currently represented by three bacterial species: *O. oeni*, *Oenococcus kitaharae*

(Endo and Okada, 2006), and *Oenococcus alcoholitolerans* Badotti et al. 2015. *O. oeni* bacteria are by far the most studied species due to their ability to perform malolactic fermentation during the alcoholic fermentation of cider or wine. Furthermore, they are tolerant of low pH and high levels of ethyl alcohol and sulfur dioxide, and they produce low concentrations of biogenic amines [55]. *O. oeni* contribute to aroma and flavor characteristics, particularly fruity and floral notes associated with volatile esters synthesized during fermentation. They also contribute to cheesy, creamy, and yogurt-like notes associated with ethyl lactate and 2,3-butanediol synthesized during malolactic fermentation. This species was first named “*Leuconostoc oenos*” based on its morphological and phenotypic similarity to members of the genus *Leuconostoc* [23]. However, this species is distinguished by its ability to grow at low pH and, unlike other *Leuconostoc* species, has a long, independent phylogenetic history. This led to its reclassification into the genus *Oenococcus* in 1995 [83].

Bacteria of the *Pediococcus* genus act as a bacterial contaminant in cider. They inhibit yeast growth and increase titratable acid concentrations. They also degrade the flavor and aroma of cider, creating a buttery/rancid odor by synthesizing high concentrations of diacetyl. Moreover, they form a viscous polysaccharide slime (“threads”), causing spoilage. Finally, these bacteria produce large quantities of lactic acid, imparting an overly harsh flavor.

Leuconostoc and *Lactobacillus* bacteria can have both positive and negative effects on cider quality. They are commonly responsible for the formation of cheesy, buttery notes and the loss of fruity flavors.

The positive effect of *Leuconostoc* and *Lactobacillus* bacteria is in converting malic acid into lactic acid under the action of cinnamoyl esterase. This makes the flavor of cider less sharp and fresh and more full-bodied and rounded. The formation of diacetyl in moderate concentrations adds creamy notes to cider’s aroma and flavor. In addition, when malolactic fermentation is complete, cider becomes more stable during subsequent bottle storage.

However, in uncontrolled fermentation, some *Leuconostoc* and *Lactobacillus* species can cause cloudiness, viscosity, and spoilage in cider. These bacteria can produce diacetyl and acetoin, as well as 4-ethylcatechol, 4-ethylphenol, and 4-ethylguaiacol from caffeic, *p*-coumaric, and ferulic acids, respectively, which causes off-odors. Moreover, *Leuconostoc* and *Lactobacillus* can produce acetic acid and other byproducts that cause off-flavors in ciders.

Genus *Acetobacteraceae*. Acetic acid bacteria of the genus *Acetobacteraceae* are Gram-negative, motile or non-motile rods that do not form spores. These bacteria produce acetic acid from ethanol through two sequential catalytic reactions of membrane-bound alcohol dehydrogenase and aldehyde dehydrogenase. Furthermore, acetic acid bacteria can oxidize various sugars and sugar alcohols, such as D-glucose, glycerol, and D-sorbitol. These

oxidation reactions are called “oxidative fermentations” because they involve incomplete oxidation of alcohols or sugars accompanied by an accumulation of oxidation products in large quantities. Acetic acid bacteria are divided into two genera, *Acetobacter* and *Gluconobacter*. *Gluconobacter* species catalyze highly active ethanol or D-glucose oxidation reactions, including the oxidation of sugars such as D-gluconic acid, D-sorbitol, and glycerol. In contrast, *Acetobacter* species have a highly active ethanol oxidation system but lack enzymes for oxidizing sugars [60, 84]. *Acetobacter pasteurianus* (Hansen 1879) Beijerinck 1916 bacteria are used in the production of apple cider vinegar [85].

Mold fungi. The natural microflora of apple fruits is also represented by mold fungi of the genera *Penicillium*, *Aspergillus*, *Botrytis*, *Monilinia*, *Alternaria*, *Diplodia*, and others. Most of them are concentrated on the surface and in the core of apple fruits. Mold fungi, particularly *P. expansum* [86], can produce a mycotoxin (patulin) which has a toxic effect and poses a serious threat to consumer health [1]. They should therefore be studied not only for their impact on the sensory properties of ciders, but also for their safety indicators. Moreover, mold fungi can disrupt biochemical processes, including enzymatic catalysis, in fresh apple must during and after alcoholic fermentation [63]. *A. alternata*, *D. seriata*, and *M. fructigena* fungi can significantly degrade the quality of cider and its sensory properties, especially aroma, by creating synthetic or chemical flavors. Their presence during apple must fermentation leads to increased concentrations of volatile acids, higher alcohols, and esters. Therefore, it is crucial to wash and inspect apples before crushing to prevent substandard raw materials (damaged skin, external defects, as well as broken, wormy, or deformed apples) from being processed into cider.

Knowing how specific microorganisms affect cider quality is vital for controlling fermentation and reducing the risk of spoilage or unpredictable changes in the beverage. In particular, it is important to know the species composition of the microflora, the microbial counts and activity, to decide on the best use of raw materials and ensure the product’s consistent and predictable quality.

Methods for detection and identification of yeasts.

Detecting spoilage yeasts in fermented beverages is a key factor in ensuring their quality and safety. The metabolic activity of some yeast species can cause irreparable damage to the products, resulting in significant economic losses for cider and wine producers [73]. Numerous methods for detecting spoilage yeasts have been proposed over the years. They have evolved significantly, from time-consuming and labor-intensive culture tests to rapid, highly sensitive molecular and metagenomic analysis methods (Table 2).

Traditional microbiological and selective methods.

Culture methods using nutrient media have long been the mainstay of microbiological control. For example, *Brettanomyces* have been isolated by utilizing special selective media, including lysine agar, media containing

antibiotics (cycloheximide), and media with a high alcohol content and low pH [87].

Autochthonous yeasts are isolated from the surface of apple fruits, grapes, cider, and wine by plating onto standard agar nutrient media (yeast extract-based glucose agar, Czapek agar, Sabouraud dextrose agar, malt extract agar, chloramphenicol-chlortetracycline agar, etc.) followed by colony selection and phylogenetic identification [88].

However, culture methods have significant drawbacks: long duration (from 24 h to several weeks), low sensitivity (many anaerobic bacteria do not grow on standard media or require special conditions), limitations (they do not detect viable, but non-culturable microorganisms in a “dormant” state), and the risk of false-positive results due to the growth of other yeasts [89]. Therefore, culture methods are often supplemented by molecular genetic (PCR) and chromatographic methods.

Direct microscopy, direct epifluorescence filtration method, and fluorescence in situ hybridization (FISH).

Direct microscopic observation is used to count yeast cell populations exceeding 10^6 cells/mL. Yeasts are often stained with methylene blue, methylene violet, trypan blue, rhodamine B, or fluorescent dyes [90]. This rapid staining can be used in cider and wine production to assess cell viability in microbial cultures.

The direct epifluorescence filtration method is a reliable alternative to direct microscopy. This method uses fluorescent dyes for total counts or detection of viable cells. For example, 4,6-diamidino-2-phenylindole stains DNA and can be used for total cell counts, while propidium iodide stains the DNA of cells with damaged membranes, which makes it useful for quantifying yeasts and assessing their viability during fermentation, as well as in the end products [99]. Commercial LIVE/DEAD BacLight kits (Molecular Probes, USA) or their analogs can be used to determine the viability of active dry yeast after rehydration. These kits include SYTO9, which stains all cells regardless of their viability, and propidium iodide, which stains damaged cells and masks the fluorescence of SYTO9, thus allowing for total counts and those of viable cells. Wine yeasts can also be detected using FISH. FISH combines the sensitivity and precision of molecular methods with direct visualization of microorganisms in their natural habitat. It is based on the use of specific nucleic acid probes containing 15–30 nucleotides and labeled with various fluorescent dyes (fluorescein, Texas Red, tetramethylrhodamine, carbocyanine, and others).

Flow cytometry. Flow cytometry has much greater potential than microscopic methods and can be used for automated detection of fluorescent signals [100]. It is widely used to analyze heterogeneous microbial populations at the single-cell level, providing morphological and physiological data. This highly automated, high-resolution method can be used to rapidly detect and quantify microorganisms in beverages, assess their viability and activity, and monitor their fermentation activity [101].

Table 2 Methods for detection and identification of yeast in cider

Method	Description	Advantages	Disadvantages	Application in cider production	Reference
Culture methods	Selective growth on specialized nutrient media	Reliability, obtaining pure cultures for further analysis, quantitative results	Duration, low sensitivity, limitations, risk of false positives due to growth of other yeasts	Isolation of <i>Saccharomyces</i> and non- <i>Saccharomyces</i> monoculture isolates from apple fruit washings, fermenting apple must, and cider samples	[37, 87]
Polymerase chain reaction	Amplification of specific DNA fragments	High speed, sensitivity, specificity	Need for expensive equipment and qualified personnel. Detecting only target taxa. Species-specific primers are designed to isolate specific targets, which limits the number of species that can be detected in a single sample	Rapid identification of <i>Saccharomyces</i> and non- <i>Saccharomyces</i> during fermentation	[87]
High-throughput sequencing	Sequencing of marker gene amplicons	Evaluation of the entire microbial community, monitoring changes, establishing correlations with metabolites	Failing to provide direct functional information, semi-quantitative in nature	Studying the influence of apple variety on the microbiota in spontaneous fermentation	[67, 91]
Hi-C metagenomics	WGS + chromosome spatial conformation capture	Obtaining high-quality MAGs, assembly at the chromosome level, autonomous circular DNA molecules inside the cell, differentiation of closely related species	Complex and expensive sample preparation requiring high computing power	Reconstruction of complete genomes of autochthonous <i>Brettanomyces</i> and <i>Saccharomyces</i> strains from spontaneously fermented cider	[92]
Fourier transform infrared spectroscopy	Irradiating yeast with infrared light of varying wavelengths. Cell components absorb the light and create specific absorption profiles	Identification of yeast down to the genus, species and strain by comparing the obtained profiles with the database	Expensive FTIR spectrometers requiring skilled maintenance; strong absorption of radiation by water complicating the analysis of aqueous solutions; complex sample preparation; sensitivity to contamination; peak overlapping in complex mixtures	Differentiation of strains of <i>Saccharomyces cerevisiae</i> , <i>Saccharomyces bayanus</i> Sacc., 1895, <i>Brettanomyces bruxellensis</i> Kuff. & van Laer (1954), <i>Hanseniaspora guilliermondii</i> Pijper, and <i>Hanseniaspora uvarum</i> (Niehaus) Shehata, Mrak & Phaff (1956).	[93–96]
Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)	Identification of specific protein patterns. Cell extracts or whole cells are placed in a chemical matrix and ionized with a laser. The ionized microbial molecules migrate to a detector through a charged field in a vacuum tube, generating a mass spectrum	A “fingerprint” unique to each microorganism can be compared to a database of known species for rapid identification at the genus and species level	High cost; matrix peak overlapping with small molecules; and limitations in distinguishing isomers	Identification of <i>Hanseniaspora uvarum</i> (Niehaus) Shehata, Mrak & Phaff (1956), <i>Metschnikowia pulcherrima</i> Pitt & M.W. Mill. (1968), <i>Saccharomyces cerevisiae</i> , <i>Schizosaccharomyces pombe</i> Lindner, 1893, and <i>Torulaspora delbrueckii</i> (Lindner 1895) Lindner 1904 at the species level. Creation of a database of yeasts isolated from vineyards and wineries	[97, 98]

Flow cytometry makes use of fluorescent dyes but has advantages over FISH probes or fluorescent antibodies. The modern market offers a wide variety of fluorescent dyes for analyzing cell populations. Propidium iodide is used as a membrane permeability marker to assess cell membrane integrity under unfavorable fermentation conditions [102]. Fluorescein diacetate can detect esterase activity in metabolically active cells and is used to assess yeast viability in both must and wine [103]. Fluorescein diacetate stains dead (yellow-green) and metabolically active (red) cells differently. It can be used to quantify cells and assess the physiological state of the yeast population. Flow cytometry combined with FISH probes has been used for the automated detection and quantification of *Saccharomyces*, non-*Saccharomyces*, and spoilage yeasts in wines [104]. The use of polyclonal fluorescently labeled antibodies has demonstrated high efficiency for flow cytometric differentiation of *S. cerevisiae* from other yeast genera during natural must fermentation, as well as for the detection and quantification of *Brettanomyces* in wine in less than 2 h [100]. However, fluorescently labeled antibodies are expensive, and their instability limits their use in winemaking.

Identification based on molecular methods. The development of molecular methods has been a breakthrough in detection speed and specificity. By providing accurate identification of microbial communities, they challenge the reproducibility of traditional identification methods, whose accuracy may depend on the physiological state of the cells. In contrast, molecular methods allow for direct genome analysis, regardless of the physiological state of the cells [105].

Among culture-independent methods, polymerase chain reaction (PCR)-based methods are widely used to detect and identify microorganisms in their ecosystems through nucleic acid analysis. Their major advantage is in extracting the total microbial DNA (or RNA) directly from the samples without culturing, which eliminates any biases that might be caused by enrichment or selective culture media. While DNA analysis typically aims to quantify microbial cell populations and assess their diversity, RNA analysis aims to identify metabolically active populations. Nucleic acid amplification can be achieved by using universal or species-specific primers. Universal primers amplify virtually all microbial DNA/RNA present in the analyzed ecosystem, thereby expanding the number of microbial targets that can be identified. In contrast, species-specific primers are designed to isolate specific targets, and their use limits the number of species that can be detected in a single sample. Nucleic acid amplification is usually combined with subsequent methods aimed at detecting, analyzing, and quantifying the resulting amplicons. These methods can be applied after the completion of amplification (post-amplification methods) or during amplification, as in the case of quantitative PCR. Indeed, PCR-based methods have advanced targeted analysis and profiling of microbial communities. However, all of them require DNA/RNA extraction and amplification of PCR primers,

and biased amplicon partitioning may limit the understanding of a microbial community [106].

Methods for detecting differences at the nucleic acid level can be divided into four groups: 1) direct detection using gel electrophoresis; 2) hybridization-based detection; 3) detection based on PCR amplification and gel electrophoresis; and 4) rDNA sequencing. Prior to any analysis, DNA or RNA must be isolated from yeast cells on the surface of apple fruits or from cider samples.

Direct detection by gel electrophoresis. For separation, DNA molecules and fragments are embedded in agarose or polyacrylamide gels and exposed to an electric field. Two methods are widely used: one for separating smaller DNA fragments obtained by cutting DNA with restriction enzymes; and the other for separating larger DNA molecules (whole chromosomal DNA) using an alternating (pulsed) electric field.

Mitochondrial (mt)DNA Analysis by Restriction Fragment Length Polymorphism (RFLP). Due to the pronounced polymorphism of *Saccharomyces* strains, mtDNA analysis by RFLP has proven useful for studying yeast biodiversity and monitoring population changes during fermentation [107, 108]. Pulsed-field gel electrophoresis allows the separation of large (chromosome-sized) DNA molecules. This method has been widely used for identification and typing, but due to complex sample preparation, lengthy electrophoretic separation, and expensive equipment, it has given way to other, simpler but no less objective methods. Furthermore, although pulsed-field gel electrophoresis is highly reproducible, the high frequency of karyotypic changes in yeasts necessitates the use of specific hybridization probes if this method is used for species identification. In particular, this method has enabled the description of electrophoretic karyotypes for several *Saccharomyces* species and other yeast genera used in cider production and winemaking, such as *Dekkera/Brettanomyces* and *Hanseniaspora/Kloeckera*.

Karyotyping is widely used for yeast differentiation and monitoring the diversity and development of species and strains during fermentation. Currently, its application is primarily limited to karyotypic studies.

Hybridization-based detection. DNA hybridization is an indirect method for identifying microorganisms that uses simple probes or microarrays to study similarities between whole genomes [109]. Hybridization can support other detection, identification, and typing methods, particularly those related to PCR. Under certain conditions, DNA hybridization can be used on its own. Single-stranded nucleic acid molecules are treated with a short labeled oligonucleotide sequence (i.e., a DNA probe) and allowed to combine to form a hybrid. Hybridization can be detected directly, by using fluorescent labels, or indirectly, by using enzyme reporters. In general, target nucleic acid molecules are immobilized on a membrane either after separation on an electrophoretic gel (i.e., Southern blotting) or from solutions (spot blotting).

Hybridization using nuclear DNA sequences (DNA-DNA reassociation) has become a standard procedure

in yeast taxonomy and identification [110], although it appears to have become less important in recent years. DNA is rarely used without hybridization to a probe. DNA probes provide specific identification and subtyping, which depend on the specificity of the oligonucleotide sequence used. Specific DNA probes can be based on conserved coding sequences, such as single-copy and polymerase chain metabolic genes. Variable sequences are also useful for typing, such as insertion elements, retrotransposons, repeated microsatellite or minisatellite sequences, or even synthetic oligonucleotides. Species-specific probes can be based on characterized sequences identified by PCR amplification [111].

Detection based on PCR amplification and gel electrophoresis. PCR amplification most often uses sequences in (mt)DNA and ribosomal (r)DNA genes (PCR ribotyping), repetitive regions of genomic DNA, and nuclear genes. These sequences may contain species-specific regions or universal sequences characteristic of all yeasts or fungi.

PCR products can be analyzed after separation by agarose gel electrophoresis or restriction fragment length polymorphism, as well as sequenced to confirm species identification.

rDNA sequencing. Sequencing is the most accurate method of analysis after PCR. This method has become a standard technique in molecular research with the advent of PCR-based direct DNA sequencing technologies that utilize automated instruments (e.g., the ABI Prism capillary electrophoresis sequencer) and gene databases for sequence comparison [112]. Over the past two decades, sequencing has become the most reliable method for identifying yeast species. Complete and partial rRNA gene sequences are most widely used in taxonomic studies and for establishing phylogenetic relationships [113]. Currently, yeast classification is based on the analysis of rDNA sequences, in particular the ITS1-ITS2 domains and D1/D2 regions of 26S rDNA. Although commercial systems are available, direct sequencing in the laboratory is still too expensive and labor-intensive for routine use in industrial settings. Typically, PCR products obtained from yeast isolates are sent to a specialized gene sequencing laboratory for identification within 1–2 days.

High-throughput sequencing (HTS) technologies (amplicon sequencing of 16S rRNA genes for bacteria and ITS for fungi) have become a powerful tool for studying the taxonomic diversity of, and changes in, microbial communities in spontaneous fermentations. They enable correlations between microbiota composition, apple variety, and aroma profile [67]. Studies have revealed a high diversity of bacterial (up to 40 genera) and fungal (up to 48 genera) communities in cider.

High-throughput sequencing. HTS is increasingly used to study the microbial ecology of various environments (e.g., grapevines) without cultivation. With HTS, scientists can characterize the microbial biodiversity of a target matrix using PCR-based ITS phylotyping (metabarcoding) or metagenomics using whole-genome sequencing.

Metabarcoding utilizes universal primers targeting rDNA and amplifying short DNA sequences with high taxonomic resolution (barcodes). PCR is followed by HTS of the amplicons using next-generation sequencing platforms. Metagenomics using DNA fragments differs from metabarcoding in that it relies on untargeted sequencing of all microbial genomes present in a sample.

In more detail, the DNA extracted from all components of a microbial community is sheared into small fragments, which are then sequenced. By sequencing the complete genome of all microorganisms present, the metagenomics approach using DNA fragments collects information on the entire gene set of all community members. Therefore, it can be used to identify both the taxonomic composition and the functional potential of microbial communities, as well as to obtain whole-genome sequences [114]. The main next-generation sequencing platforms vary in sequencing chemistry, sequence length (reads), the number of sequences per run, and cost.

High-throughput sequencing yields a vast number of reads, whose multivariate statistical analysis can give a comprehensive description of microbial biodiversity [115]. Specifically, the obtained data can be subjected to taxonomy-independent or taxonomy-dependent analyses. In taxonomy-independent analysis, genetic diversity is organized by grouping the sequences with the sequences of related species. These groups of sequences must correspond to taxonomic clades and are operational taxonomic units [116]. In taxonomy-dependent analysis, reads are identified according to their similarity to reference sequences in taxonomically annotated databases [117].

High-throughput sequencing has demonstrated that grape and wine microbiota have regional patterns that influence the fermentation behavior and wine characteristics. Furthermore, the microbial composition of grapes can be used as a biomarker for predicting final wine quality [118]. These studies continue to expand our knowledge of microbial diversity during fermentation and help us identify sources of microbial contamination [119].

Instrumental methods. Fourier transform infrared spectroscopy is another method for yeast identification and characterization. This method is based on irradiating yeast with infrared light of different wavelengths. Different cellular components (e.g., proteins, fatty acids, polysaccharides) absorb the light and create specific absorption profiles. By comparing the obtained profiles with a relevant database, scientists can identify the genus, species, and even strain of yeasts [120]. Although commonly applied in many fields, this method has been underutilized in ecological studies of cider or wine. Some studies used it to differentiate the strains of *S. cerevisiae*, *S. bayanus*, and *Brettanomyces bruxellensis* Kuff. & van Laer (1954) [121], as well as the strains of *Hanseniaspora guilliermondii* Pijper and *H. uvarum* [120]. The disadvantages of FTIR spectroscopy include strong absorption by water, which complicates the analysis of aqueous solutions, complex sample preparation, sensitivity to contamination, and peak overlapping in complex

mixtures. The method also requires strict path length control in the cuvette for quantitative analysis. Finally, FTIR spectrometers, especially high-resolution spectrometers, are expensive instruments that require skilled maintenance.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a reliable, rapid, inexpensive, and high-throughput method for identification and classification of microorganisms. This method is based on the identification of specific protein patterns, mainly ribosomal proteins. For this, cell extracts or whole cells are placed in a chemical matrix and ionized with a laser. The ionized microbial molecules migrate toward a detector through a charged field in a vacuum tube and generate a mass spectrum. This is a kind of “fingerprint” unique to each microorganism that can be compared with a database of known species for rapid identification at the genus and species levels [97]. MALDI-TOF MS was used to identify *H. uvarum*, *M. pulcherrima*, *S. cerevisiae*, *S. pombe*, and *T. delbrueckii* species [122]. In another study, scientists created a database of yeasts isolated from vineyards and wineries based on MALDI-TOF MS spectra. This database is based on an extensible open-source platform for MALDI data processing and analysis using statistical methods [123]. Kits are also available for identification of microorganisms by mass spectroscopy (Vitek MS MALDI-TOF).

CONCLUSION

Our review revealed that knowing the species composition, quantity, and activity of microflora in raw materials (e.g., apples) is key to producing products with predictable quality. This necessitates research to identify and quantify microbial populations, as well as their

metabolic networks, on the surface of harvested apples. Such research may help us understand what factors affect the formation of beneficial microbial communities and how these communities can be manipulated to produce high-quality ciders. In the long term, it will enable us to replace imported active dry yeasts with domestic preparations.

In this review, we analyzed the diversity of microorganisms involved in cider production and the main methods for identifying *Saccharomyces* and non-*Saccharomyces* yeasts. However, what remains understudied is how the varietal characteristics of apple trees and their place of origin affect the genetic and species diversity of microorganisms. Also, further research is needed to assess the enzymatic activity of non-*Saccharomyces* and their role in the sensory and physicochemical properties of ciders, as well as the main biotechnological processes. Finally, we need to improve the existing methods, and develop new techniques, for identifying microflora and assessing its effect on the quality of the beverage as early as during fermentation. All this calls for further research into the diversity of microflora involved in cider production.

CONTRIBUTION

A.A. Khrapov managed the project; N.M. Ageeva developed the study concept and design; E.A. Al-Nakib, E.V. Lobodina, and Yu.N. Chernyavskaya collected, analyzed, and interpreted literature data; A.A. Shirshova wrote and edited the article; E.V. Globa revised the text, the tables, and the references, as well as prepared the article for publication. All the authors approved the final version of the article and are responsible for its integrity.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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Additional information about the authors

Anastasia A. Shirshova ORCID 0000-0003-1428-5935; eLIBRARY SPIN 6175-5869
 Natalia M. Ageeva ORCID 0000-0002-9165-6763; eLIBRARY SPIN 3807-3865
 Anton A. Khrapov ORCID 0000-0001-6436-1970; eLIBRARY SPIN 6182-5762
 Ekaterina A. Al-Nakib ORCID 0000-0003-0855-1176; eLIBRARY SPIN 7630-6098
 Julia N. Chernyavskaya ORCID 0009-0003-0504-9997; eLIBRARY SPIN 6046-2433
 Elena V. Lobodina ORCID 0000-0002-3580-2316; eLIBRARY SPIN 5485-5500
 Ekaterina V. Globa ORCID 0000-0002-6890-3076; eLIBRARY SPIN 8576-4008