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BIOTECHNOLOGICAL ASPECTS OF SOUR BEER PRODUCTION

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Abstract. The article presents a review of current biotechnological approaches to the production of sour beer styles, with a focus on the use of lactic acid bacteria (LAB) as a means of controlled acidification. Sour beer styles have gained wide popularity among consumers due to their vibrant flavor profiles, which are shaped by the activity of various groups of microorganisms. In particular, LAB play a key role in this process, as they are capable of producing lactic acid, thereby lowering the wort's pH and ensuring the microbiological stability of the final product. The paper describes the features of different technological approaches to the production of sour beers: spontaneous fermentation (traditional lambics), mixed fermentation (American sour ales), kettle souring, primary souring, and less common methods such as sour mashing or pre-acidification of malt. Special attention is given to the advantages of the kettle souring method, which allows acidification prior to primary fermentation, ensuring consistent quality, reduced production time, and simplified process control. Based on literature analysis, it was established that among LAB, the most promising for use in brewing are representatives of facultatively and obligately heterofermentative LAB. The most extensively studied LAB species to date include *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, and *Levilactobacillus brevis*, which are capable of growing in beer wort, rapidly lowering its pH, and contributing to the unique organoleptic profile of the beverage. At the same time, the article emphasizes the limited number of LAB strains suitable for use in sour beer production and highlights the need for further research aimed at the isolation, selection, and biochemical characterization of new technologically valuable cultures, as well as the optimization of their cultivation conditions.

Key words: brewing, microbiota, lactic acid bacteria, Lactobacillaceae, kettle souring, mixed fermentation, lactic acid fermentation.

Introduction. Formulation of the problem

Brewing can be considered one of the oldest branches of the food industry [1]. It is known that for most of its history, brewing was a domestic or small-scale commercial activity [2]. Recent archaeological research focused on brewing indicates that the production of the first fermented beverages resembling beer began at the onset of the "Neolithic Revolution" and the development of agriculture, dating back to the 9th millennium BCE [3]. Evidence of ancient beer production has been found across all continents, particularly in Asia (Mesopotamia, China) [4], North Africa (Egypt) [5], South America (Peru) [6], Europe, and others [7].

The primary prerequisite for the beginning of beer and other fermented beverage production is considered to be the cultivation of various cereal crops, particularly wheat, barley, and rice [1,3]. Other important conditions included the availability of suitable vessels, the acquisition of experience in grain processing (storage, germination), and the presence of a controlled energy source [1]. Analyzed samples of ancient beer contained various ingredients that, to some extent, differ from those typical today. Fermentation was initiated using fruits, honey, fruit juices, oak bark, grapes, and similar materials [3,8]. Additionally, evidence points to the use of bread and sourdoughs for wort fermentation, indicating a parallel

development of baking and brewing practices [9]. Such components helped lower the pH of the wort and enriched the grain substrate with yeasts, which directly fermented the main carbohydrates, leading to the accumulation of alcohol [3].

The further development of brewing can be attributed to the greater availability of many cereal crops and the low cost of this raw material, especially in comparison to grapes, which were widely used in winemaking [1-3]. Climatic factors and the spread of beer and related fermented beverages in Northern Europe, particularly in Scandinavia, laid the foundation for key traditions in modern brewing, such as the use of hops [3]. It was hops and the hopping process that contributed to the popularization of beer by enabling the standardization of flavor characteristics and the extension of shelf life, thereby increasing the product's marketability [3,8].

An important milestone in the further development of brewing is considered to be the Beer Purity Law, or *Reinheitsgebot* in German, which restricted the ingredients used in beer production to malt, water, and hops; yeast was later added to the regulation [1]. It can be said that this document was one of the first to establish quality requirements for the final beverage and to formally define the term "beer" [2,3,8]. Additionally, the emergence of bottom fermentation in beer production laid the foundation for the development of lager beer styles [1].

The further development of brewing has been closely linked to various scientific fields and advancements, including: biochemistry – through the study of the nature of fermentation and the conversion of sugar into alcohol, as well as the investigation of enzymes, particularly amylases; and microbiology – through the identification and isolation of pure yeast cultures such as *Saccharomyces pastorianus* (*S. carlsbergensis*) [10], *S. cerevisiae*, and *S. Bayanus* [1,8,11]. Other important innovations involved the establishment of industrial-scale beer production processes, including the use of thermometers and saccharometers [3,8], steam engines, standard brewing equipment, as well as the founding of educational institutions (such as the Institute of Brewing and Distilling, UK) and professional journals (The Journal of the Institute of Brewing, The Australian Brewers' Journal, Bayerische Bierbrauer) focused on brewing [2]. Alongside scientific discoveries, the production of various beer styles developed actively – for instance, the emergence of top fermentation and ale styles such as porter is well documented [1,2].

The aforementioned discoveries played a crucial role in establishing the main organoleptic properties of beer, standardizing the beverage and its production stages, and in the subsequent development and popularization of beer in the 20th century, which gave momentum to the scaling of the industry. As a result, beer has become one of the most popular alcoholic beverages in the world today [1, 12]. However, catering to a wide consumer base with a recognizable and uniform flavor (as in the case of a single beer style) led to a loss of unique organolptic characteristics [3]. Today, the typical (traditional industrial/high-volume) beer production process follows a standardized protocol that includes: malt milling, mashing, lautering, boiling (with hopping), cooling, fermentation, conditioning (maturation), filtration, and packaging [8,12].

Traditionally, one of the most important characteristics of beer as an alcoholic beverage has been its pronounced organoleptic properties and the wide variety of styles. However, the standardization and reduction of flavor diversity contributed to the gradual revival of craft brewing [13]. Historically, small breweries – or, according to modern terminology, craft breweries – were the original producers before the industrialization of the sector [14]. The renewed development of craft beer production was also influenced by economic factors and the higher quality of the final product, which can be achieved more easily under small-scale production conditions [3,15]. The concept of craft beer production is not uniformly defined worldwide. However, under the definition adopted in the United States, craft breweries are typically characterized by small size, independence, and traditional methods [15]. In the U.S., the trade group Brewers Association defines a craft brewery as one with: an annual production of up to 6 million barrels (704,086,800 liters), no more than 25% of the brewery owned or controlled by a beverage alcohol industry member that is not itself a craft brewer, and beer characterized by sensory qualities derived from fermentation using traditional or innovative brewing ingredients [15,16]. In other countries with well-developed craft brewing sectors (such as Germany, Canada, the United Kingdom, Australia, the Czech Republic, Italy, and Ukraine) [17], similar criteria are known to apply, though they may vary depending on national beer consumption volumes [15].

Overall, craft brewing is highly popular today, and according to economic indicators, the industry is experiencing an unprecedented period of growth [14]. The strong interest among consumers is driven by the opportunity to produce specialty beer styles, new styles, or traditional styles using alternative raw materials, novel ingredients, or with higher alcohol content in the final product [14,18,19]. The implementation of technological production standards and the preservation of traditional recipes allow craft breweries to produce more than 150 recognized beer styles to date [15,18]. Although craft brewing encourages the creation of new styles, each produced beer style is expected to reflect its characteristic organoleptic features and physiological-biochemical properties, most of which have been shaped historically. To standardize the defining features of

beer styles, the Beer Judge Certification Program (BJCP) Style Guidelines are often used [20].

Historically, beer styles are categorized based on the type of fermentation: top-fermented – ales, bottom-fermented – lagers, and mixed fermentation [2]. Other parameters commonly used to assess and classify beer styles include alcohol content, bitterness level, color, original gravity, raw materials used, and organoleptic properties. The classification of beer styles is shown in Figure 1.

Among all types of beer, the most popular is the standard or international lager [21]. In addition, ale styles are gaining popularity today, particularly stouts, pale ales, wheat beers, porters [15,22], as well as fruit beers, specialty, and sour beers [15,23].

Although sour beer styles currently account for a small share of the craft beer market, there is growing interest in producing many popular styles that fall under the categories of European sour ale and specialty beer, including Berliner Weisse, Lambic, Fruit Lambic, Gueuze, Gose, and others [24]. Sour beer styles have many distinctive features, with one of the main ones being their organoleptic properties and sour taste, which result from the spontaneous or intentional use of lactic acid bacteria (LAB) during their production [12]. Despite the long history of brewing, including sour beer styles, the microbiota of many of these styles remains largely unexplored. Establishing effective technological regimes and selecting LAB strains for their controlled use in sour beer production remains a relevant challenge in biotechnology and brewing.

Thus, **the aim** of this review is to highlight the main technological methods used in the production of sour beer styles, analyze the studied microbiota characteristic of these styles, describe the application of non-conventional yeasts, and evaluate the use of LAB of various origins for the production of sour beer styles.

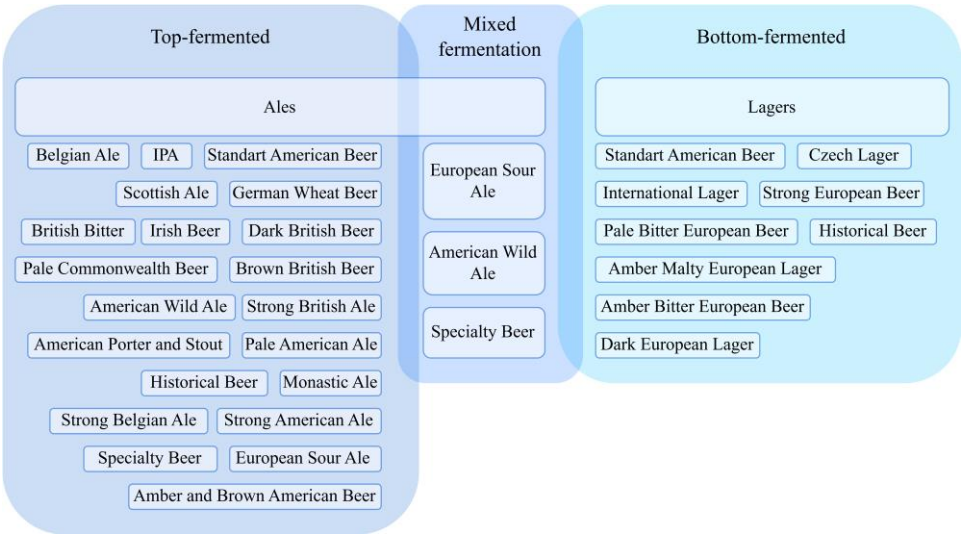

Fig. 1. General classification of the main categories of beer styles [2, 20]

Analysis of recent research and publications

1. Sour beer styles

1.1. Overview of typical representatives

Sour beer styles belong to the ale category (Fig. 1) and are produced either using top-fermenting yeasts or through spontaneous (mixed) fermentation [12,20]. The main distinguishing feature of sour beer styles is the use of LAB, which is atypical for the vast majority of beer styles and is generally considered a contaminating factor in the industrial production of lagers and many craft beer styles [25]. Examples of such contaminating LAB include *Levilactobacillus brevis*, *Fructilactobacillus lindneri*, *Secundilactobacillus paracollinoides*, *Lactiplantibacillus plantarum*, *Lentilactobacillus buchneri*, *Loigolactobacillus coryniformis*, and *Pediococcus damnosus* [25-27]. The formation of turbidity and the synthesis of lactic and acetic acids pose challenges in the production of typical, non-sour beer styles, as they impair the sensory properties of the product [25,27]. However, in the case of sour beer styles, these metabolic activities of LAB are usually desired and necessary to achieve the specific sensory characteristics typical for the style [25].

Sour beer styles are usually classified under BJCP categories 23 European Sour Ale and 28 American Wild Ale. A comparison of typical styles is presented in Table 1.

Table 1 – Comparison of the main characteristics of sour beer styles [20]

Characteristic		European Sour Ale							American Wild Ale			
		Style										
		Berliner Weisse	**Flanders Red Ale**	**Oud Bruin**	**Lambic**	**Gueuze**	**Fruit Lambic**	**Gose**	**Beer with Brett**	**Mixed Fermentation Sour Beer**	**Wild Specialty Beer**	**Straight Sour Beer**
Consistency	Body	Light	Medium, enhanced by tannins	From medium to medium-full	From light to medium-light	From light to moderately light		From medium-light to medium-full	Light			
	Carbonation	High	From slight to medium	From low to moderate	Uncarbonated or moderate	High	From sparkling to almost none	From very high to very sparkling	From medium to high	From moderate to high	High	From moderate to high
	Acidity	Crisp	Prickly	-	From medium to high, without sharp astringency	From medium to high		From moderate to restrained	-			Crisp
Typical ingredients	Malt	Pilsner, wheat	Vienna or Munich	Pilsner, dark caramel, small amounts of colored malts	Pilsner			Pilsner, wheat	Depend on the base style			Light (Pilsner, wheat, combinations)
	Hops	-	Continental with low alpha acid content		Old hops			-				-
	Other components	-	Optional natural or artificial sweeteners	-	Unmalted wheat		Fruits, optionally artificial or natural sweeteners	Salt, coriander				Optional light sugar
Typical microorganisms		Saccharomycetes and Brett LAB	Saccharomycetes, Brett, LAB	Saccharomycetes, LAB	Spontaneous fermentation microorganisms			Saccharomycetes, LAB	Brett	Saccharomycetes, LAB, and Brett		Saccharomycetes, LAB, or acid-producing yeasts
Key indicators	OG	1,028-1,032	1,048-1,057	1,040-1,074	1,040-1,054			1,040-1,060	1,036 - 1,056	Depend on the base style		1,048-1,065
	IBU	3-8	10-25	20-25	0-10				5-12			3-8
	FG	1,003-1,006	1,002-1,012	1,008-1,012	1,001-1,010		1,000-1,006	1,000-1,010	1,006 - 1,010			1,006-1,013
	SRM	2-3	10-17	15-22	3-7		5-6	3-7	3-4			2-3
	ABV, %	2,8-3,8	4,6-6,5	4,0-8,0	5,0-6,5		5,0-8,0	5,0-7,0	4,2-4,8			4,5-7,0
Fermentation type		Top-fermented			Mixed				Top-fermented	Mixed		Top-fermented

- – not specified in the source, OG – original gravity, IBU – International Bitterness Units, FG – final gravity, SRM – Standard Reference Method degrees, ABV – alcohol by volume, Brett – representatives of the *Brettanomyces* genus.

1.2. Technological features of sour beer styles production

One of the main characteristics of sour beer styles is the low pH level of the beverage – ranging from 3.0 to 3.9 – resulting from high concentrations of organic acids [12]. The elevated acid content is achieved through the involvement of LAB or other microorganisms capable of acid production. Depending on the specific style and production scale, various acidification methods may be employed, as illustrated in Figure 2 [12,24,28].

One of the oldest methods is spontaneous fermentation, which is traditional for Belgian styles (Lambic, Gueuze, Fruit Lambic) [24], as well as for American mixed fermentation wild ales [20,24,28]. In this acidification method, hopped or unhopped wort is left overnight to cool after boiling. Then, in the case of traditional lambics, the wort – naturally inoculated with "wild" microorganisms – is transferred to barrels and stored at low temperatures (from 0°C to 25°C) for a period ranging from 4-8 months to up to 3 years [25,28]. Another variation of spontaneous fermentation involves barrel-aging beer, a technique used for producing specialty or newly developed beer styles. Aging in wooden barrels (usually oak) allows for the creation of sour beers with novel and unconventional organoleptic characteristics [28]. The main drawback of producing sour beer styles using spontaneous fermentation is considered to be product instability [12].

More recent and currently widespread technological methods that accelerate the production of sour beer styles include sour malting, sour mashing, kettle souring, primary souring, and mixed fermentation [24, 28]. The use of these methods is made possible by the availability of isolated pure cultures of LAB and yeasts. The main differences among these technological approaches lie in the stage of the process and the specific microorganisms responsible for souring the substrate [12,28]. Methods such as sour malting [28], sour mashing [29], and kettle souring typically employ various LAB strains [12,24,28], which are added to the malt, mash, and wort respectively. In the case of mixed fermentation, mixtures of microorganisms – particularly yeasts and LAB – are used, and they are introduced either directly into the wort or into hopped wort, depending on the recipe and the beer style [24,28]. Primary souring differs from the others in that it involves the use of a relatively new category of yeast strains capable of acid production. In this case, cultures are introduced directly into the hopped wort [30].

The methods described above represent modern alternatives for the production of sour beer styles; however, they cannot fully replace the original recipes used for styles produced through spontaneous or "wild" fermentation. The organoleptic properties of such styles are more complex due to the prolonged fermentation process and the metabolic activity not only of LAB but also of many other microorganisms [24,28].

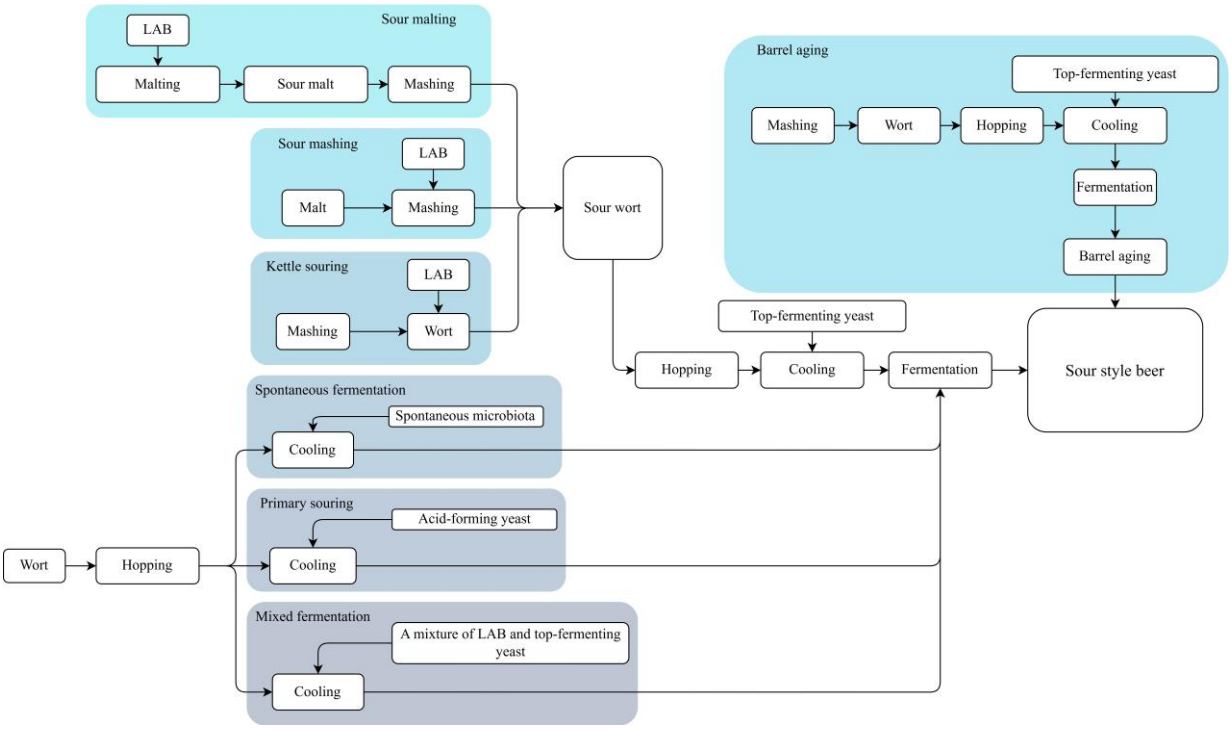

Fig. 2. A generalized diagram of known methods for producing sour beer styles [12, 28]

1.3. Microbiota of sour beer styles

Although there is growing interest in studying the microbiology of various sour beer styles, the majority of publications focus on Belgian spontaneously fermented beer styles, particularly lambics. Research into the composition of microbiota and the metabolic processes occurring during spontaneous fermentation in the production of sour beer styles allows for a better understanding of the impact of microorganisms on the organoleptic characteristics of the final product.

Nowadays it is established that spontaneously fermented styles are characterized by distinct fermentation phases, which include the *Enterobacteriaceae* phase, primary fermentation, acidification, and maturation phases [11]. While the division of the process into phases is important, from a microbiological perspective, the development of microorganisms occurs simultaneously. The identification of phases is instead associated with the dominant microbiota present at a specific time and the corresponding biochemical processes.

For example, it is known that the *Enterobacteriaceae* phase may overlap with the primary fermentation phase [31,32]. As the name suggests, the typical microorganisms of the first phase are members of the *Enterobacteriaceae* family. In lambics, identified species include *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Hafnia protea*, *H. alvei*, *Citrobacter freundii*, *Serratia ureilytica*, *Serratia* spp., *Proteus mirabilis*, *E. agglomerans*, *E. ludwigii*, *E. cloacae*, *E. Mori* [11,31,33,34]. This phase typically lasts from 3 to 12 weeks [11,31]. In the case of American coolship ale, identified species include *Kl. oxytoca*, *Kl. pneumoniae*, *E. agglomerans*, *E. ludwigii*, *E. cloacae*, *E. mori*, and *Sr. ureilytica* [11,32]. Yeast species associated with this phase include *Candida krusei*, *Pichia fermentans*, *P. kluyveri*, *Cr. keutzingii*, *Rhodotorula mucilaginosa*, *Rh. mucilaginosa* [31,32].

The next stage is the primary fermentation phase, which is characterized by a yeast-dominated microbiota. In lambics, the dominant yeast species include *Saccharomyces cerevisiae*, *S. bayans*, *Candida* spp., *Cryptococcus* spp., *Pichia* spp., *Torulopsis* spp, *Brettanomyces* spp. [11,31,33,34]. In American ales, *Brettanomyces bruxellensis* and *S. cerevisiae* are the predominant yeasts [32]. The duration of this phase varies, but is typically reported to range from 2 to 9 weeks [11]. According to [28], cycloheximide-resistant yeasts are also active at this stage. It is known that during the primary fermentation phase, acetic acid bacteria (AAB) and LAB, such as *Pediococcus damnosus* [31], also become active in biochemical processes, marking the transition to the next phase – acidification [11,28]. In lambics, the typical microbiota of the acidification phase includes *Pediococcus* spp. (*P. damnosus*) [11,31,35], *Acetobacter* spp. (*A. lambici*, *A. orientalis*, *A. fabarum*, *A. pasteurianus*) [31,36], *Gluconobacter* spp. (*Gl. cerevisiae*, *Gl. cerinus*) [31], *Lactobacillus* spp [11,37]. In American coolship ales, a broader diversity of LAB from the *Lactobacillaceae* family has been identified, including *Lactobacillus delbrueckii*, *L. curvatus*, *Levilactobacillus brevis*, and *Apilactobacillus kunkeei* [32]. For both styles, *B. bruxellensis* was found to be dominant during the acidification phase [11,31,32,34]. The maturation phase is characterized by refermentation and continued metabolic activity of the dominant microbiota established in the previous phases. In lambics, these include *B. bruxellensis* and *P. damnosus* [11,31,34], *A. pasteurianus*, *A. lambici* [35], *B. custersianus* [35,38]; in American coolship ales – *Phaffomyces opuntiae*, *Pediococcus* spp., *Limosilactobacillus fermnetum*, *Lv. brevis*, *Ap. kunkeei*, *Fructilactobacillus lindneri* [32].

The acidification phase is particularly important, as it has a significant impact on the final organoleptic properties of the beverage during spontaneous fermentation [12,25,28]. Based on the established microbiological characteristics of typical representatives of sour beer styles, it can be stated that the primary acidification processes are carried out by microorganisms from three groups: LAB, AAB, and yeasts capable of synthesizing organic acids [12,28,39]. Due to their metabolic activity, microorganisms from these groups can participate in spontaneous fermentation and be used to create controlled acidification conditions, particularly in technological approaches such as kettle souring and primary souring [12,28].

1.3.1. Lactic acid bacteria

Historically, LAB are defined as a ubiquitous, heterogeneous group of bacteria united by common metabolic properties – namely, the ability to synthesize lactic acid as the main end product of fermentation (metabolism or carbohydrate utilization) [40,41]. From a biological diversity standpoint, LAB are considered based on taxonomic, genetic, and phenotypic differences, and are also differentiated depending on their source of isolation and tolerance to extreme conditions [42]. LAB representatives share such phenotypic characteristics as: Gram-positive staining, non-sporulating, catalase-negative, acid-tolerant, and facultative anaerobic. In addition, LAB are regarded as non-pathogenic microorganisms with the status of "Generally Recognized As Safe" (GRAS) [40].

Phylogenetically, the group currently includes representatives of the families *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, and *Streptococcaceae*, which belong to the phylum *Bacillota*, as well as the genus *Bifidobacterium* of the phylum *Actinomycetota* [42,43]. Although the group comprises many families and species with distinctive metabolic characteristics, in the context of brewing, the greatest attention is given to members of the family *Lactobacillaceae*, such as the genera *Lactobacillus*, *Lactiplantibacillus*, *Pediococcus*, among others. LAB are a widespread group of microorganisms occupying numerous ecological niches. Dairy products are a classical source of LAB isolation [44], but other common sources include fermented plant materials [45],

fermented juices [46], meat products [47], sourdoughs [9], etc. It is important to note that the modern classification of the *Lactobacillaceae* family considers not only the morphological and biochemical characteristics of the species but also their source of isolation and natural habitat [48].

The *Lactobacillaceae* family is quite heterogeneous in its physiological and biochemical properties. All representatives are characterized by facultative anaerobic or aerotolerant metabolism, the ability to grow at pH levels ranging from 4 to 8, at temperatures from 2°C to 53°C, as well as the fermentation of various carbohydrates – depending on the species – resulting in the production of lactate or other byproducts [41,49]. Carbohydrate utilization and, consequently, lactic acid biosynthesis constitute the central metabolic pathway in members of this family, meeting the cells' energy demands through homo- or heterofermentative lactic acid fermentation, as illustrated in Figure 3 [50].

Based on the presence of enzymatic systems and the ability to utilize pentoses, LAB are classified as obligate homofermentative, facultatively heterofermentative, and obligate heterofermentative [43,51]. The *Lactobacillaceae* family includes representatives that exhibit all fermentation types, depending on the species [49]. In homofermentative fermentation, sugars – primarily hexoses – are fermented via the Embden-Meyerhof-Parnas (glycolytic) pathway, resulting in lactic acid (lactate) as the final product [40,43,51]. Homofermentative fermentation is typical for genera such as *Pediococcus*, *Lactobacillus*, *Amylolactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, etc. [40,48,51]. Heterofermentative fermentation by LAB typically utilizes the phosphoketolase and pentose phosphate pathways for carbohydrate metabolism. This type of fermentation involves not only hexoses but also pentoses (e.g., fructose) and disaccharides (e.g., sucrose, maltose). The process results in the production of lactic acid (lactate) along with byproducts such as ethanol and acetate [40,51]. The heterofermentative pathway is characteristic of genera such as *Limosilactobacillus*, *Levilactobacillus*, *Lactiplantibacillus*, and others [40,43,52]. Other metabolites that may be formed during homo- or heterofermentative fermentation, or through subsequent conversion of lactate, include acetate and acetoin, or 1,2-propanediol and acetate, respectively [51,52]. Additionally, alternative products derived from pyruvate include formate (formic acid), ethanol and acetate, succinate, and acetoin, which may also be associated with homofermentative metabolism [40,51,52].

Members of the *Lactobacillaceae* family possess the ability to utilize not only monosaccharides (glucose, fructose), but also the majority of oligosaccharides (isomaltose, maltodextrins, other α-glucans), as well as polysaccharides (starch, amylopectin, glycogen). This capacity to metabolize such compounds is considered a survival and adaptation strategy of LAB to various natural environments [54]. Amylolytic activity in *Lactobacillaceae* is supported by the presence of amylase genes in species such as *Lpb. plantarum* [55-58], *Lacticaseibacillus manihotivorans* [57], *L. amylovorus* [57,58], *L. amylolyticus* [59], *Lmb. fermentum* [60]. Furthermore, activity of maltose phosphorylase, extracellular α-glucosidases, and glucanotransferases has been reported in species such as *Fructilactobacillus sanfranciscensis* and *Lmb. fermentum* [61], *L. acidophilus* [62,63], *Lcb. Rhamnosus* [64], and *Lmb. reuteri* [65], respectively.

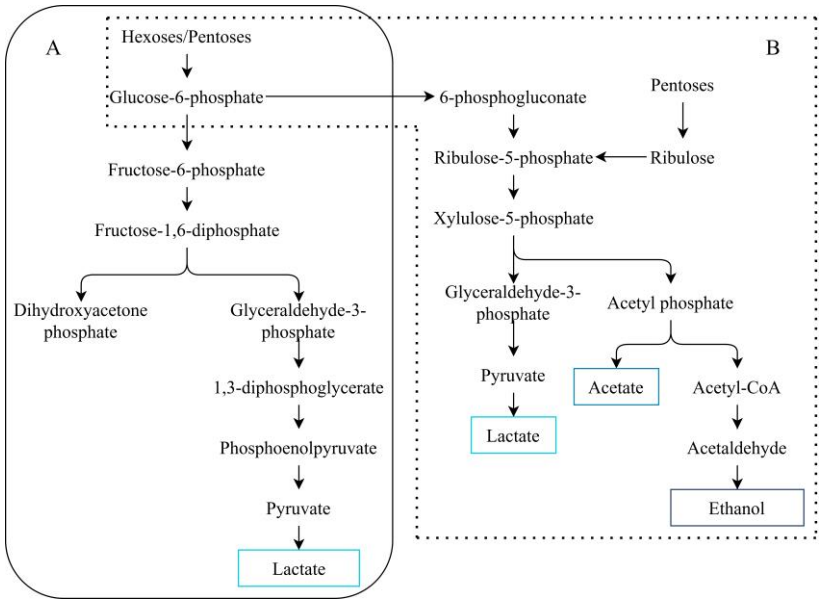

Fig. 3. Carbohydrate metabolism of LAB. A – homofermentative pathway; B – heterofermentative pathway. The end products of each pathway are indicated within the boxes [51, 53]

A typical feature of LAB, including members of the family *Lactobacillaceae*, is the presence of a proteolytic system comprising cell-envelope proteinases, endopeptidases, aminopeptidases, specific aminopeptidases, and proline-specific peptidases [40,66]. It is important to note that in the case of LAB, the proteolytic system plays a vital role in their viability, as the majority of representatives are auxotrophic for numerous amino acids [53,66,67]. At present, cell-envelope proteinases are considered among the best-characterized enzymes within the LAB proteolytic system. Recent studies have identified a wide variety of such proteinases, with the most well-known and characterized enzymes being: PrtP (*Lcb. paracasei* subsp. *paracasei*) [67,68], PrtB (*L. delbrueckii* subsp. *bulgaricus*) [69], PrtH (*L. helveticus*) [70], PrtR (*Lcb. rhamnosus*) [71], PrtL (*L. delbrueckii* subsp. *lactis*) [72]. Based on their origin, these proteinases are believed to have the ability to hydrolyze milk proteins such as casein, although some studies also report their capacity to degrade meat proteins and those of plant origin [66]. The end products of protein and amino acid metabolism from various sources are commonly volatile compounds that contribute to the aromatic profile of specific fermented products, for example: ethanol, dimethyl sulfide, dimethyl trisulfide, thioesters, cresol, acetaldehyde, and certain biogenic amines [40,43,73]. Thus, it can be concluded that protein metabolism in LAB is not only essential for sustaining viability and adaptation to environmental conditions but also significantly influences the organoleptic characteristics of fermented foods [67,74].

A novel area of LAB research involves the discovery of phenolic compound metabolism, including phenolic acids, flavonoids, tannins, and other dietary phenolics [75, 76]. The presence of enzymes involved in the transformation of phenolic and polyphenolic compounds is considered a key factor in the adaptation of LAB to plant-based substrates [75]. The most extensively studied species in this context include *Lpb. plantarum* [77, 78], *L. johnsoni* [79], *Lvb. brevis* [80], and *Lmb. antri* [81]. The metabolism of phenolic compounds plays an important role in shaping the organoleptic profile of certain products, and therefore, the use of LAB can be beneficial in the production of fermented foods derived from plant materials [76].

1.3.2. Acetic acid bacteria

In addition to LAB, another group of microorganisms that can contribute to beer acidification is AAB [25]. Although, unlike LAB, they are less common and of lower industrial relevance, they still play an important role in the production of spontaneously fermented beer styles [12,28]. Overall, AAB belong to the family *Acetobacteraceae*, which includes 21 genera [82]. The most well-studied representatives are species of the genera *Acetobacter*, *Gluconobacter*, and *Komagataeibacter* [83]. Morphologically, AAB are heterogeneous, though rod-shaped cells are typical of the mentioned genera[83-85]. AAB share similar physiological and biochemical characteristics: they are Gram-negative, strictly aerobic, mesophilic microorganisms. Although they are generally considered obligate aerobes, some researchers report the existence of microaerotolerant strains, particularly those isolated from draft beer [25]. The most typical and optimal temperature range for growth is 25°C to 30°C, with a maximum growth temperature of 37°C. The optimal pH range for growth is 5.0–6.5, although growth can also occur at lower values (as low as 3.0–4.0) [84,85]. Additionally, AAB are catalase-positive and oxidase-negative [83,84]. AAB are also widely distributed in nature and inhabit various ecological niches. The most typical sources include vinegar [86], fermented beverages such as kombucha [87, 88], plant materials, low- or alcoholic beverages, honey, bees, and occasionally soil and water [89].

From the perspective of nutritional requirements, AAB are considered to be non-fastidious microorganisms, especially when compared to the previously discussed group of LAB [84]. In particular, the majority of AAB strains are capable of utilizing various carbon sources such as glucose, arabinose, fructose, mannitol, galactose, ribose, xylose, sorbitol, and ethanol [84,86]. A defining physiological trait of AAB is their oxidative metabolism, which enables the aerobic oxidation of alcohols (ethanol), alditols or sugar alcohols (mannitol, sorbitol) into the corresponding organic acids, aldehydes, and ketones [83,84,86,90]. A generalized scheme of the main metabolic pathway of AAB is presented in Figure 4.

The most well-known end product of AAB metabolism is acetic acid. However, the enzymes involved in their oxidative metabolism can convert substrates such as D-gluconic acid, fructose, sorbitol, glucose, myo-inositol, sorbose, and sorbonose into metabolic precursors including 2,5-diketogluconate, 5-ketofructose, sorbose, glucono-δ-lactone, 2-ketoinositol, sorbone, and 2-ketogulonic acid, respectively [92,93]. In addition, the presence of membrane-bound acetaldehyde dehydrogenase enables the oxidation not only of ethanol but also of various other alcohols, such as propanol, butanol, isobutanol, glycerol, and pentanol, into the corresponding acids: propionic, butyric, isobutyric, glyceric, and valeric acid [90-92]. Once the primary carbon sources – carbohydrates and alcohols – are depleted, the accumulated acids can be fully oxidized to carbon dioxide and water [84,94].

Another metabolic feature of AAB, particularly characteristic of the typical species *Gluconobacter oxydans*, is its ability to synthesize vitamin C from glucose through intermediates such as 2-keto-gulonic acid and L-sorbose [90].

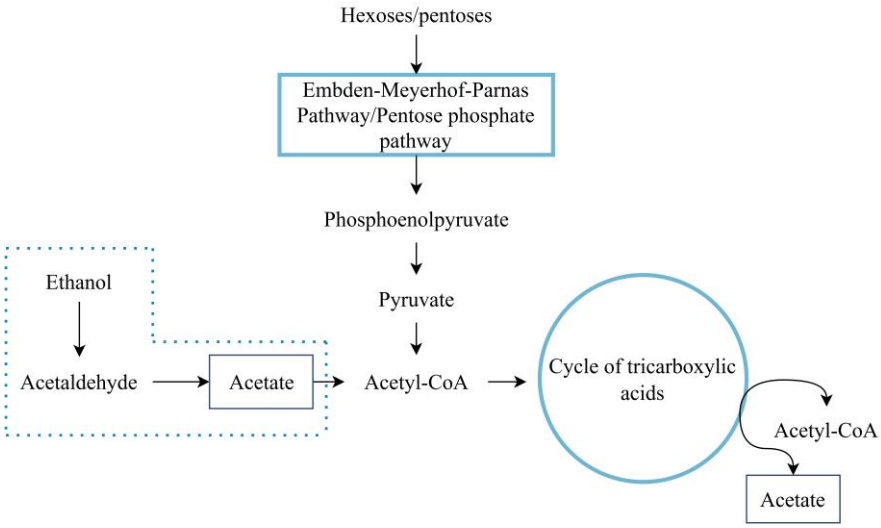

Fig. 4. Carbohydrate metabolism of AAB. The end products of metabolism are indicated within the boxes. The dotted line represents the ethanol oxidation pathway [83, 91]

Like LAB, AAB – particularly members of the genera *Acetobacter* and *Gluconobacter* – also possess the ability to synthesize aromatic compounds. For example, the production of esters such as ethyl acetate is well-documented and is attributed to the presence of intracellular esterases, which are characteristic of the majority of *Acetobacter* species [95,96]. Another aromatic compound, more typically associated with LAB, is acetoin, which is also actively produced by AAB representatives [95]. The study [97] demonstrates the ability of *A. malorum* and *G. oxydans* to generate a wide range of aromatic and phenolic compounds during wine vinegar production. Among the dominant phenolic compounds were 5-hydroxymethylfurfural, 3,4-dihydroxybenzaldehyde, ethyl gallate, syringaldehyde, and p-coumaric acid; while the most abundant volatile and aromatic compounds included ethyl acetate, isobutyl acetate, ethyl butyrate, isoamyl acetate, and phenethyl acetate [97]. The intense oxidative metabolism of AAB, along with their capacity to produce not only acetic acid and intermediate metabolites but also a wide spectrum of phenolic, aromatic, and volatile compounds, can significantly influence the organoleptic properties of the final product.

1.3.3. Yeasts

In addition to the well-known acid-producing microorganisms from the LAB and AAB groups, recent decades have seen growing interest in yeasts capable of accumulating not only ethanol but also organic acids such as acetic and lactic acid [39, 98]. The primary species exhibiting the ability to synthesize organic acids and actively acidify the medium are non-*Saccharomyces* yeasts, particularly representatives of the genera *Brettanomyces*, *Candida*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Torulospora*, *Yarrowia*, *Lachancea*, etc. [99].

1.3.3.1. *Brettanomyces bruxellensis*

The most typical representative of non-*Saccharomyces* yeasts that plays an important role in brewing – particularly in spontaneously fermented beer styles such as lambic – is *Brettanomyces bruxellensis* [28,100]. *B. bruxellensis* belongs to the subphylum *Saccharomycotina*, family *Saccharomycetaceae*, and shares many nucleotide sequences with *S. cerevisiae* [101]. The most common sources of its isolation include wine, beer, bioethanol, and various fermented beverages such as kombucha and tequila [100,101]. This species demonstrates a high capacity for long-term survival in various industrial environments, which is attributed to its morphological and physiological traits, including the ability to form biofilms, pseudomycelium, and chlamydospore-like structures [101].

The nutritional requirements and energy metabolism characteristics of *B. bruxellensis* are less well-characterized compared to the model organism *S. cerevisiae*, though there are specific features unique to *B. bruxellensis* strains [101]. For instance, such atypical traits include variability in growth rate among isolates, high adaptability to "harsh" nutrient environments – particularly those lacking glucose or ammonium – and significant metabolic diversity within the species [100-102]. The primary metabolic pathways of representatives of the species are typical for yeasts, with carbohydrate fermentation proceeding via the Embden-Meyerhof pathway and the tricarboxylic acid cycle [103]. The most notable metabolic feature is the synthesis of acetic acid, which is usually considered a beneficial antagonistic trait against non-resistant microorganisms [100,101]. Biochemical studies focused on the synthesis of ethanol and acetic acid indicate that *B. bruxellensis* tends to accumulate more acetic acid than ethanol, and the level of aeration has been shown to influence the accumulation of these metabolites [104].

Although in winemaking *B. bruxellensis* is considered an undesirable member of the microbiota due to its activity leading to off-flavors [100,105], in brewing the species plays an important role in lambic production, as it positively contributes to the sensory profile of the finished beer [100]. Owing to these properties, the species' ability to produce secondary metabolites is currently well studied [101, 106]. However, it is important to note that the synthesis of secondary metabolites, like other biochemical traits, may vary depending on the cultivation medium and the source of the isolate [100]. The biochemical characteristics of the species include the production of volatile phenolic compounds (4-vinylguaiacol, 4-vinylphenol, 4-ethylguaiacol, and 4-ethylphenol) [106], volatile fatty acids (isovaleric acid, amyl octanoate, butyric acid, heptanoic acid) [107], organic acids (citrate, malate, succinate, phenylacetic acid), and phenols (vanillin, 4-ethylferol) [108]. A more common trait, shared with AAB and other yeasts, is the ability to synthesize volatile esters such as ethyl acetate, ethyl lactate, ethyl caprate, and ethyl caprylate [106-108].

1.3.3.2. *Lachancea thermotolerans*

Another acid-producing non-*Saccharomyces* yeast species is *Lachancea thermotolerans*, which has gained popularity due to its distinctive metabolic characteristics [98]. *Like B. bruxellensis*, *Lc. thermotolerans belongs to the family Saccharomycetaceae* [109]. It is the most extensively studied species within the genus *Lachancea* [98,110]. *Lc. thermotolerans* occupies diverse ecological niches, with wine, juices, and other high-sugar sources being the most common isolation environments, indicating its tolerance to high osmotic pressure (up to 60%) [110]. Similar to *B. bruxellensis*, *Lc. thermotolerans* isolates exhibit physiological differences depending on their source of isolation [98,110,111].

Lc. thermotolerans exhibits a metabolic profile similar to that of *S. cerevisiae* and *B. bruxellensis*, including the Embden-Meyerhof pathway, the pentose phosphate pathway, and the tricarboxylic acid cycle. However, its most distinctive feature, which makes this yeast species unique, is its ability to accumulate not only ethanol but also lactic acid. The authors of study [109] propose that the species' ability to synthesize lactic acid is a sign of the anthropization of *Lc. thermotolerans* – a gradual adaptation to fermentation under stress conditions through the regulation of the cell's redox potential. Other major metabolic products include small amounts of acetic acid and acetaldehyde [110]. Study [99] highlights the ability of *Lc. thermotolerans* strains to accumulate higher levels of organic acids such as formate, pyruvate, and oxalate compared to other non-*Saccharomyces* yeasts.

As with *Saccharomyces* and non-*Saccharomyces* yeasts, *Lc. thermotolerans* possesses the ability to synthesize a wide range of aromatic compounds, which is well-studied due to its application in winemaking and its potential use in the production of other beverages. Typical volatile compounds produced by *Lc. thermotolerans* include ethyl acetate, ethyl lactate, 1-propanol, isobutanol, isoamyl alcohol, 2-phenylethanol [112-114], acetoin, higher alcohols [110,115], diethyl acetal [113,114], etc.

The main distinction between the examined yeasts lies in their elevated synthesis of organic acids – acetic acid for *B. bruxellensis* and lactic acid for *Lc. thermotolerans*. Both yeast species are characterized by the accumulation of volatile aromatic compounds, such as ethyl lactate and ethyl acetate. Although volatile compounds significantly influence organoleptic properties, most studies analyzing their synthesis in yeasts indicate that similar aromatic compounds can also accumulate during fermentation by typical *S. cerevisiae* strains [99].

The discussed microorganisms each have specific advantages and disadvantages in terms of organoleptic characteristics and production conditions for sour beer styles. In particular, the group of AAB can be involved in the production of spontaneously fermented styles (lambic, gueuze) [93-95]. However, the formation of acetic acid as a primary metabolite may negatively impact the beverage's organoleptic profile, imparting a sharp, bitter, vinegar-like aroma [95,116]. A key advantage of *Brettanomyces* spp. yeasts is their ability to simultaneously synthesize ethanol and acetic acid as primary metabolic products. Nevertheless, excessive acetic acid synthesis – similarly to AAB – limits their application. Additionally, there are reports of potential risks associated with *Brettanomyces* spp. due to their ability to produce biogenic amines, which can cause undesirable physiological effects when consumed in high concentrations, as well as their resistance to cycloheximide [100]. The lack of fully characterized metabolites potentially produced by non-*Saccharomyces* yeasts is also considered a drawback for their industrial use [98]. Yeasts *Lc. thermotolerans* are considered promising, as they have the ability to simultaneously synthesize lactic acid and ethanol and have a positive impact on organoleptic properties, creating a fruity organoleptic profile of the beverage [114,117]. Another advantage is their broader applicability, including for mixed fermentation and primary souring processes. However, a limitation is their lower levels and slower accumulation of lactic acid compared to LAB representatives [99,113].

2. Use of LAB for producing sour beer styles

The most versatile microorganisms used in the production of sour beer styles are LAB, which combine several important features: rapid accumulation of high concentrations of lactic acid, compatibility with technological processes that ensure organoleptic and microbiological stability of the beer (such as mixed fermentation and kettle souring), a positive impact on the sensory profile of the beverage, and improvements in certain physicochemical properties [12,28].

Currently, only a limited number of industrially available species are used for the production of sour beer

styles, provided by manufacturers such as Lallemand (Canada) – *Lpb. plantarum*, *L. Helveticus* [118], White Labs (USA) – *Lvb. brevis*, *L. delbrueckii*, *Lpb. plantarum*, *Ltb. buchneri*, *Ltb. hilgardii* [119], Wyeast (USA) – *Ltb. buchneri*, *Lvb. brevis*, *P. damnosus* [120], BSI (USA) – *L. delbrueckii*, *Lvb. brevis*, *P. Damnosus* [121]. The limited number of LAB strains available for brewing has encouraged the development and application of new species for producing sour beer styles. One of the main selection criteria for new strains is their ability to grow in wort, acidify it, and positively influence the sensory properties of the final product. The results of studies focused on LAB selection for the production of various sour beer styles are presented in Table 2.

The majority of LAB strains used consist of facultatively (*Lpb. plantarum*, *Lcb. paracasei*, *Lpb. pentosus*) and obligately heterofermentative species (*Lmb. fermentum*, *Ltb. buchneri*, *Lvb. brevis*). Depending on the applied technological process, the LAB species, and the temperature and duration of acidification, the resulting final pH values may vary, though higher pH values are typically observed for obligately heterofermentative species. Studies indicate a positive effect from the use of LAB, including enhanced fruity aroma in the final product [123,126] and improved foam stability [125] in the case of certain strains.

Other studies have explored the use of LAB in the production of chapalo, a spontaneously fermented sorghum beer. The study [134] demonstrates the effectiveness of applying *Lmb. fermentum* and *P. pentosaceus* under kettle souring conditions. Since the vast majority of LAB are microorganisms with probiotic properties, their integration into beer production has led to the consideration of beer as a potential delivery matrix for probiotics, or probiotic beer, which may exert beneficial health effects on consumers, as reported in studies [135-138]. Research has also focused on accelerating the acidification process of beer wort or enhancing sour beer formulations through the addition of raffinose-family oligosaccharides [139], as well as passion fruit and peach juices [133].

Table 2 – Overview of recent studies on the use of LAB in brewing

Microorganisms used	Wort pH		Technological regime	Souring temperature, °C	Souring time, h	Reference
	Before souring	After souring				
Lcb. paracasei	5.65	3.27	Kettle souring	30	48	[122]
P. pentosaceues		3.41				
Leuconostoc mesenteroides		3.50				
L. gasseri	5.85	3.66		37	12	[123]
L. bulgaricus	-	3.45		42	72	[124]
Lpb. plantarum	4.50	3.43		40	40	[125]
Lmb. fermnetum		3.17				
Lpb. pentosus		3.44				
Ltb. buchneri	5.70	4.10		18	24	[126]
Lvb. brevis	5.50	3.71		30	48	[127]
Lpb. plantarum		3.43				
Lvb. brevis	5.50	3.60		20	168	[128]
Lvb. brevis, *S. cerevisiae* US-05		3.70	Mixed fermentation	20	168	
Lcb. paracasei subsp. *paracasei*, *S. cerevisiae* US-05	5.80	4.83		37	48	[129]
Lpb. plantarum, *S. pastorianus*	-	3.89-4.56		20	120	[130]
Lcb. paracasei subsp. *paracasei*, *S. cerevisiae* US-05	5.90	4.30		37	24	[131]
Lpb. plantarum	5.98	3.23	Fermentation using LAB	Variable temperature: 38 °C for the first 41 hours, 21 °C for the remaining time	378	[132]
Lcb. rhamnosus		3.18				
L. delbrueckii		4.01				
Lvb. brevis		3.42				
Ltb. buchneri		3.42				
Companilactobacillus alimentarius		3.25				
Lcb. paracasei subsp. *paracasei*	5.80	4.35	Primary fermentation	37	24	[133]
Streptococcus thermophilus		5.19				

Conclusion

Brewing is an ancient branch of the food industry that has undergone significant changes over time. In the 21st century, craft brewing has experienced rapid development, leading to increased demand for unconventional styles, particularly sour beers, which require the involvement of non-traditional microorganisms capable of biosynthesizing organic acids.

Sour beer styles can be produced using various technological approaches, including kettle souring, primary souring, spontaneous fermentation, and mixed fermentation. It has been established that the use of the controlled kettle souring method allows for achieving consistent sensory characteristics while reducing the impact of uncontrolled wort microbiota.

Compared to microorganisms typically involved in spontaneous fermentation, LAB offer the greatest advantages, including rapid acidification, safety, and versatility of use. They can contribute both to the acidification process and to the subsequent microbiological stability of the beverage.

The literature analysis revealed that the most commonly used strains in research are *Lpb. plantarum*, *Lcb. paracasei*, and *Lvb. brevis*; however, the practical application of these cultures in kettle souring, mixed fermentation, or primary souring regimes has not been sufficiently studied.

The issue of a limited set of well-characterized LAB strains adapted to the conditions of beer wort – particularly its high sugar content, bitter compounds, and ethanol – remains relevant.

Further research should focus on investigating the physiological and biochemical properties of promising lactic acid bacteria strains, optimizing the conditions for their cultivation in beer wort, and evaluating their ability to produce desirable or undesirable metabolites that affect the quality of the final product.

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БІОТЕХНОЛОГІЧНІ АСПЕКТИ ВИРОБНИЦТВА КИСЛИХ СТИЛІВ ПИВА

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Анотація. У роботі представлено огляд сучасних біотехнологічних підходів до виробництва кислих стилів пива з акцентом на використання молочнокислих бактерій (МКБ) як засобу контрольованого підкислення. Кислі стилі пива набули широкої популярності серед споживачів завдяки своєму яскравому смаковому профілю, що формується за участі різних груп мікроорганізмів. Зокрема, ключову роль у цьому процесі відіграють МКБ, які здатні утворювати молочну кислоту, знижуючи pH сусла та забезпечуючи мікробіологічну стабільність готового продукту. Описано особливості різних технологічних підходів до виробництва кислих стилів: спонтанного бродіння (традиційні ламбіки), змішаного бродіння (американські кислі елі), підкислення у чані (kettle souring), первинного підкислення (primary souring), а також менш поширених методів, таких як кисле затирання чи попереднє підкислення солоду. Увагу приділено перевагам методу kettle souring, який дозволяє здійснювати процес підкислення до основного бродіння, забезпечуючи повторюваність якості, скорочення часу виробництва та спрощення контролю за технологією. У результаті аналізу даних літератури визначено, що серед МКБ найбільш перспективними для використання у пивоварінні є представники факультативно та облігатно гетероферментативних МКБ. Найбільше досліджень на сьогодні приділено таким видам МКБ, як: *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei* та *Levilactobacillus brevis*, які мають здатність до росту у пивному суслі, швидкому зниженню його pH та створення особливого органолептичного профілю напою. Разом із тим, наголошено на обмеженій кількості штамів МКБ, придатних до використання в умовах виробництва кислих стилів пива, та необхідності подальших досліджень, спрямованих на ізоляцію, селекцію та біохімічну характеристику нових технологічно цінних культур, а також оптимізацію умов їх культивування.

Ключові слова: пивоваріння, мікробіота, молочнокислі бактерії, *Lactobacillaceae*, підкислення у чані, змішане бродіння, молочнокисле бродіння.