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A Review of N-Heterocycles: Mousy Off-Flavor in Sour Beer

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ABSTRACT: Beer has over 600 flavor compounds and creates a positive tasting experience with acceptable sensory properties, which are essential for the best consumer experience. Spontaneous and mixed-culture fermentation beers, generally classified as sour beers, are gaining popularity compared to typical lager or ale styles, which have dominated in the USA for the last few decades. Unique and acceptable flavor compounds characterize sour beers, but some unfavorable aspects appear in conjunction. One such unfavorable flavor is called "mousy". This description is usually labeled as an unpleasant odor, identifying spoilage of fermented food and beverages. It is related as having the odor of mouse urine, cereal, corn tortilla chips, or freshly baked sour bread. The main compounds responsible for it are N-heterocyclic compounds: 2-acetyltetrahydropyridine, 2-acetyl-1-pyrroline, and 2-ethyl-tetrahydropyridine. The most common beverages associated with mousy off-flavor are identified in wines, sour beers, other grain-based beverages, and kombucha, which may contain heterofermentative lactic acid bacteria, acetic acid bacteria, and/or yeast/fungus cultures. In particular, the fungal species *Brettanomyces bruxellensis* are associated with mousy-off flavor occurrence in fermented beverages matrices. However, many factors for N-heterocycle formation are not well-understood. Currently, the research and development of mixed-cultured beer and non/low alcohol beverages (NABLAB) has increased to obtain the highest quality, sensory, functionality, and most notably safety standards, and also to meet consumers' demand for a balanced sourness in these beverages. This paper introduces mousy off-flavor expression in beers and beverages, which occurs in spontaneous or mixed-culture fermentations, with a focus on sour beers due to common inconsistency aspects in fermentation. We discuss and suggest possible pathways of mousy off-flavor development in the beer matrix, which also apply to other fermented beverages, including non/low alcohol drinks, e.g., kombucha and low/nonalcohol beers. Some precautions and modifications may prevent the occurrence of these off-flavor compounds in the beverage matrix: improving raw material quality, adjusting brewing processes, and using specific strains of yeast and bacteria that are less likely to produce the off-flavor. Conceivably, it is clear that spontaneous and mixed culture fermentation is gaining popularity in industrial, craft, and home brewing. The review discusses important elements to identify and understand metabolic pathways, following the prevention of spoilage targeted to off-flavor compounds development in beers and NABLABs.

KEYWORDS: *mousy off-flavor, N-heterocycles, spontaneous fermentation, sour beer, tetrahydropyridines, 2-acetyl-pyrolline*

1. INTRODUCTION TO BEER BREWING AND SOUR BEER STYLE

Spontaneously controlled fermentation of food and beverages is rising rapidly. Social media groups with keywords in English "wild fermentation", "home brewing", "craft brewing", and similar have from 1 to 100 thousand members and 1−20 daily posts. Nevertheless, the academic community is also interested in fermentation and brewing topics. The scientific articles and reviews in brewing topics for publishers likewise, Elsevier, MDPI, and American Chemistry Society increased by 60−80% for the past five years (from 2018 to 2023).[1−3] Moreover, the past 10 years have shown rapid growth of craft brewers worldwide, including in Italy, Mexico, New Zealand, and Asia markets. From 2018 to 2022, craft beer made up 13% of the total beer market in the U.S. This represents over 24 billion dollars and tends to increase slowly.[4,5] When focusing on alcoholic and non/low alcoholic beer fermentation, there are mainly four different types.[6,7] Two are the most known lagers and ales, including bottom and top fermentation using one specific yeast strain. The other two types are spontaneous and mixed-cultured fermentations. In general, brewing is a process that involves the continual application of heat, usually for plant material (hot side) and its preparation for fermentation (cold side). Figure 1 represents general beer brewing flow. Grain selection and the malting process are the first and crucial steps to achieve a favorable product.[8] Typically, the mashing process starts when selected and crushed (fine or coarse) malt is mixed with water. Grist composition can be adjusted depending on the desired beer style. Different mashing temperatures and retention times activate proteolytic and amylolytic enzymes like α- and β-amylase, limit dextrinase, α-glucosidase, cysteine protease, serine protease, and dozens of others which release a certain amount of reducing sugars, peptides, amino acids, and phenolic compounds from grains endosperm and cell wall. To perform test wort for malt quality analysis, the European

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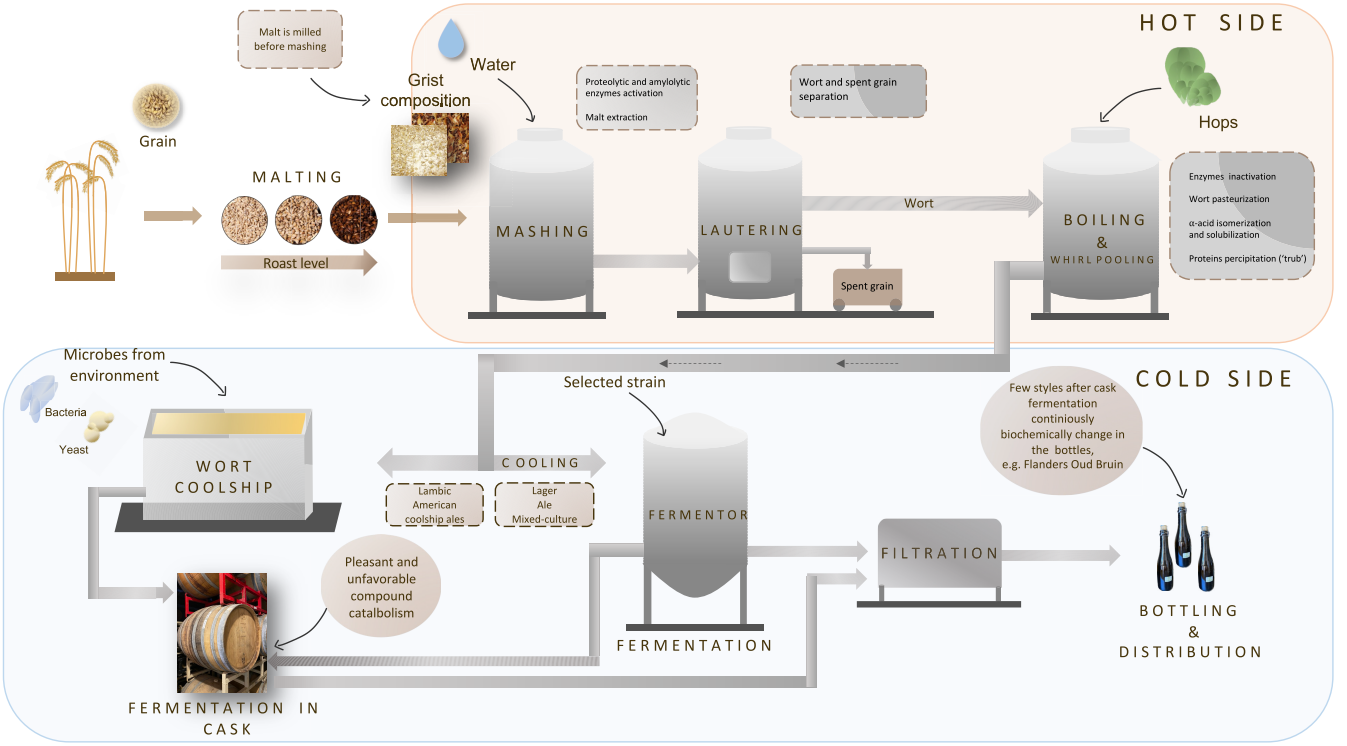

Figure 1. General brewing scheme for different beer styles.

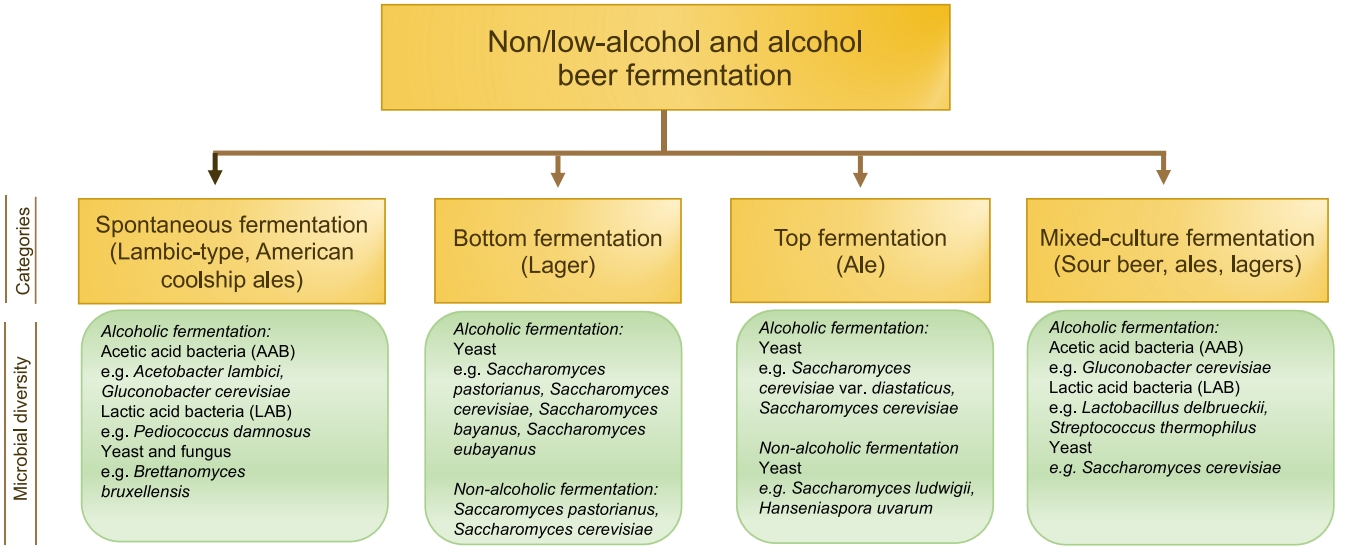

Figure 2. Beer fermentation categories and their possible microbial variety.

Brewery Convention (EBC) recommends temperature and time variation of 45 °C 30 min and 70 °C 60 min, while the Institute of Brewing (IoB) mashing method describes one temperature of 65 °C for 60 min. Commonly, mashing guidelines are around 65 °C, 68 °C, and up to 75 °C from 10 to 60 min in different breweries, depending on the beer style being produced.[9−13] Following the lautering step, it separates enriched extract, called wort, from spent grain. Wort is continuously transferred to a boiling kettle where enzymes denature, the wort is pasteurized, and isomerization and solubilization of added hops occur. The final hot sidestep ends after whirlpooling, and the cold side starts by transferring filtered and cooled wort to a fermentation vessel (lager, ale, mixed-culture beer styles) or open pool (lambic-style, American coolship ale beer). The first option is commonly used for already-known yeast or bacteria strains incorporation. Compared to lambic-style (LS) or American coolship ale (ACA) beer brewing, wild environmental microbes are collected in the open pool. Continuously after the open environmental inoculation, fermentation follows in casks and eventually in bottles, which impacts the flavor compounds significantly.

The wine industry already targeted N-heterocyclic compounds as precursors to undesirable flavors which may contribute to wine spoilage and thus economic losses.[14−18] The main scope of this review is to systemize scientific

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understanding about N-heterocyclic compounds and their formation, identify mousy off-flavor occurrence in the beer matrix, evaluate N-heterocyclic compounds identification methods, and suggest possible steps to prevent mousy off-flavor formation.

2. SOUR BEER CHARACTERISTICS

Generally, classification of four different categories can be introduced based on microbial diversity used in beer brewing (alcoholic and lon/low alcoholic) according to 2023 Brewers Association beer guidelines which describe 160 different styles[19] (Figure 2).

Sour beer involves spontaneous fermentation and mixed-culture fermentation. Spontaneous definition includes open coolship step in beer brewing and mainly two generalized styles of lambic and ACA can describe this category.[20,21] Other sour beer styles like Gueuze, Faro, Flanders, and Kriek are blended from differently aged casks beer or enriched with sugary adjuncts. Conversely, mixed-culture fermentation also fits the sour beer category. However, main deviation from spontaneous is that mixed-culture beer brewing exclude environmental microbial and yeast inoculation flow.[7,22]

The primary difference between sour and typical beers is the diversity of metabolic pathways induced by microbial population. The result is that sour beers have higher organic acid content, which decreases the sour beer pH to 3.0−3.9.[23,24] Lager and ales pH vary around 4.0−4.5.[25] Many organic acids, such as citric, gluconic, and malic acids, are identified in sour beers. However, lactic and acetic acids are the most prevalent.[26] Also, sour beers have increased succinic acid concentration, which can be in the range from 150 to 800 mg kg^{-1} and is involved in fruity aromatic esters production and have a positive flavor outcome.[23,27,28] Malolactic fermentation usually is involved while brewing a sour beer. During LS beer production, the malolactic fermentation starts only after 2−3 months of aging in the barrel. Latest LS beer production publication identified malic acid significantly decreased during the acidification stage while lactic and acetic acid concentrations increased from 1 to 5 g L^{-1}.[23] Compared with non/low alcohol, sour beers are produced as lagers or ales with adjuncts, such as cherries or other sour juices, which contributes to enhanced fruitier flavor.[29]

Other characteristics of sour beer that differentiate it from standard lagers and ales are attributed to low-calorie intake and unique flavors. The complexity of sour beers production and microbial population variety increases the diversity of beneficial secondary metabolites in the beverage matrix, e.g., prebiotics, polyphenols, minerals, and vitamins.[5,29−34] Spontaneous or mixed-culture fermentations contribute to other beneficial outcomes, such as possible probiotic bacteria occurrences, e.g. *Bacillus valencia*, isolated from Apong rice beer, which is spontaneously fermented beer.[35] A recent clinical study shows that moderate or low/nonalcoholic beer consumption (up to one drink per day/14 g alcohol for women and up to two drinks per day/28 g alcohol for men, which typically comprises one or two bottles of beer (330 mL) with 4% w/v alcohol) may positively influence the diverse human gut microbiome without significant change of chronic diseases markers.[36]

As mentioned above, this outcome is from polyphenols, minerals, vitamins, and soluble fiber, such as the prevalence of β-glucans in the beer matrix. Nonetheless, sour beer can be produced using probiotic strains such as *Lactobacillus paracasei* and, in slight process modifications, can even be viable in the product.[37] However, randomized clinical trials on beer beneficial outcomes may differ depending on the geographical manners of the human population.[38] On the contrary, non/low alcohol beer is suggested to be as effective as commercially available sports drinks or rehydration solutions with plant-based sources of B group vitamins, silica, folate, vitamin C, niacin, selenium, potassium, and polyphenols.[39,40]

3. MOLECULAR APPROACH OF MOUSY OFF-FLAVOR

Currently, there are over 15 000 craft breweries worldwide.[39] Innovation and competition among macro- and micro-breweries increased subtle variations and experimentation in malt selectivity, hop varieties, brewing, and fermentation techniques.[41−44] As a result, a wide spectrum of unique tastes and aromas has increased along with off-flavors. Likewise, onion-like off-flavor which is caused by 2-mercapto-3-methyl-1-butanol (2M3MB) and 3-mercapto-3-methyl-1-butanol (3M3MB), aldehydes, e.g., acetaldehyde with "grassy" off-flavor if concentration is above 10−20 mg L^{-1}, and mousy off-flavor associated with N-heterocyclic abundance.[45−49]

Mousy off-flavor in beer has not received as much attention as other off-flavors. However, the compounds responsible for mousy off-flavor in beer have been reported.[50−52] One of the first identifications of corny, cereal, fresh popcorn, and sour bread odor, mouse urine, and cracky off-flavor in malt and beer, which is attributed to N-heterocycles, were investigated 40 years ago.[53,54] Mousy off-flavor usually refers to tetrahydropyridines (THP), which are found in 2-ethyl-1,3,4,5,6-tetrahydropyridine (enamine form) and 2-ethyl-3,4,5,6-tetrahydropyridine (imine form) (ETHP), 2-acetyl-1,4,5,6-tetrahydropyridine (enamine form), and 2-acetyl-3,4,5,6-tetrahydropyridine (imine form) (ATHP) forms.[14,15,55,56] However, the third compounds 2-acetyl-1-pyrroline (imine form) and 2-acetyl-2-pyrroline (enamine form) (APY) are also involved in the perception of mousiness. Although the APY chemical structure affiliates from pyridines, it cannot be eliminated from a mousy off-flavor discussion. The metabolic pathway of APY is similar to ATHP and has a resemblance of similar molecular weight (111.1 g mol^{-1}) and specific ions (m/z 63/83/111).[55] These ions were determined using GC-O, GC-FTIR, and GC-MS using electron impact techniques.[57,58] Interestingly, the fourth cracky-like odor compound 2-formyl-1,4,5,6-tetrahydropyridine (FTHP) can also be involved in mousy off-flavor development in the beverage matrix. However, the presence of FTHP identification in fermented beverages is limited and has barely been described. Moreover, its stability is lower than ATHP tautomers, which implies a challenging approach in the different matrices of food and beverage.[58]

ATHP and ETHP have thresholds in water of 1.6 μg kg^{-1} and 150 μg kg^{-1}, respectively, whereas the APY threshold in water using orthonasal evaluation was 0.1−0.06 μg kg^{-1}. Scientific data on N-heterocycles content in beer samples is limited, while in spoiled wines ATHP varied from 4.8 to 106 μg kg^{-1}, and APY content increased by 75−130-fold.[59] Although THP is typically described as off-flavor, brewers sometimes initiate ETHP as a favorable aftertaste compound due to the increased popularity of mixed-culture beers.[60] Traditional Lambic beer and American coolship ales possesses a unique flavor spectrum, which if in balanced symbiosis, creates a full body, sharp, and fruity acidic, 'brett-flavor' mouthfeel experience.[61]

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Perception of ATHP, ETHP, and APY applies to their imine and enamine forms, the protonation of which is altered in acidic and neutral/alkaline media. When tasting N-heterocycle abundant beer, neutral saliva pH basifies beer pH, which follows N-heterocyclic compound protonation to a more volatile imine form. It reaches olfactory epithelium and gives a stronger and longer perception of a mousy-off flavor.[55,62]

4. MICROBIAL AND CHEMICAL PATHWAYS OF N-HETEROCYCLES FORMATION

In the beer matrix, N-heterocycles can be sourced in several ways. First, APY, ATHP, and ETHP can develop from metabolic pathways involving microbial communities. Second, pyridines and pyrrolines can be Maillard and Strecker degrading products, which are formed during the kilning of malt, especially with higher roast levels.[48,52,53,63] Due to limited studies and analytical methods as well as complex processes and variables of different beverage matrices, it is unclear which pathways play a more significant role in forming mousy off-flavor.[43−47]

Spontaneously or mixed-culture fermentations have a higher risk of developing mousy off-flavor. First, because of the higher probability of having *Brettanomyces bruxellensis* (teleomorph *Dekkera bruxellensis*) and over a dozen different *Bretanomyces* species, specific heterofermentative lactic acid bacteria (LAB) and acetic acid bacteria (AAB) prevalence during fermentation.[24] Higher viability of these microbes can play a reasonable part in mousy-off flavor development.[64] More extended fermentation timings also might be a factor for mousy off-flavor development, as this widely applies to beer, including open coolship and/or cask fermentation. During hopped wort cooling prior to fermentation, there is a higher risk of undesirable spontaneous bacteria or yeast inoculation, and every cask may have different flora, impacting metabolic pathways and making inconsistent batches. However, discerning clearly how, when, and which factors contribute to off-flavor prevalence remains to be a challenge. Conditions for spontaneous or mixed-culture fermentations are different and can differ in bottles; for example, three-year-old lambic beer and different barrel fermented Gueuze beers produced in the same technological flow had different strains. *D. bruxellensis* and *D. anomala* were identified in most bottles; *D. bruxellensis* was found in a bottle aged more than 15 years.[65]

Also, more than nine different minerals, glycolysis degradations products, and specific amino acids as precursors might be involved for N-heterocycle formation.[14] For example, proline with methylglyoxal (MGX) during alcoholic fermentation develops APY,[66,67] which alone can be favorable, but in complex with tetrahydropyridines are responsible for mousy off-flavor in wines.[55,68] However, MGX can be formed from glucose through retro-aldol condensation, which can continuously react with proline and develop ATHP during Maillard reaction and particularly redox reactions.[63,69] Proline is the second most abundant amino acid found in cereals and hence malt, which is scarcely assimilated by yeast.[16,56,70,71]

4.1. Chemical Pathways Involved in N-Heterocycles Formation. Another biochemical pathway of APY and ATHP can be a result from amino acids proline, lysine, and ornithine degradation products involved in Maillard and Strecker reactions. Targeted amino acids react with α-dicarbonyl from glucose, resulting in 1-pyrroline, α-hydroxyketone, pyrrolidine, and α-diketone, which eventually, through different metabolic pathways, develops flavory N-heterocycles.[53,72−74] Proline-rich foods are more prone to develop ATHP. Although the specific concentration of how much of amino acids needed to develop N-heterocycles has not been evaluated, some experimental media for chemically developing ATHP uses 5 g kg^{-1} of specific amino acid.[14,15,72]

THP compounds and APY can be obtained in heat-involved steps during the beer production chain. First, the high level of proline in malt, which carries through into final beer as most beer yeasts do not assimilate proline, can trigger THP development. However, the kilning stage in malting is a crucial step for flavor compound formation due to the Maillard reaction. Roasted barley tea volatiles analysis identified, that APY content varied between 0.82 μL kg^{-1} and 1.02 μL kg^{-1} (evaluated threshold was 0.053 μL kg^{-1}). Naked barley tea had higher content of APY compared with hulled barley tea.[75] Proline can be involved in flavor development and is also observed to bring light-brown color and flowery, pleasantly sweet, persimmon, and bitter taste after 14 and 24 h of Maillard reaction. In contrast, lysine in Maillard reactions contributes to a dark color and caramel-like, bitter odor.[76]

Further, higher hot side production temperatures are obtained during multistep mashing and boiling procedures.[8,77] Especially boiling step where hops are added as additional amino acid source and temperature reaches over 90 °C. Pyridines and Strecker reaction degradation products are developed after 5 min of proline−glucose interactions in heat.[78]

In general, sour beers and specifically fruity sour beers are prone to develop THP due to oxygen and minerals, such as iron divalent ion, increase.[56] Organic acids such as malic and citric acids are involved in mousy off-flavor compound development.[14,15,79] Higher content of malic acid, a metabolite of malolactic fermentation, was found after 2−6 months in barrel-aged beer, suggesting higher risks for THP to occur in the later stages of cask fermentation and beer maturation.[61,80]

4.2. Microbial Diversity and Pathways Involved in N-Heterocycles Formation. ATHP, ETHP, and APY are metabolites but not necessarily ubiquitously produced yeast and few LAB strains.[48,52,53] In particular, three main species that were linked to produce N-heterocycles in mousy off-flavor wines: one yeast strain *Brettanomyces bruxellensis* and two LAB strains *Lentilactobacillus hilgardii and Oenococcus oeni*.[14] Currently, the suggested mechanism for ATHP production by *Dekkera/Brettanomyces sp*. yeasts, combines the amino acid L-lysine and ethanol or acetaldehydes.[56,81,82] Amino acid L-lysine through enzymatic catabolism produces Δ-piperdine, which through acylase condensation reaction initiates ATHP formation.[64] A similar pathway contributes to APY development where L-ornithine is a substitute for L-lysine.[16,56] During the beer aging process, *Brettanomyces* slowly converts ATHP to ETHP by dehydrogenase enzyme, which can take from 6 up to 36 months.[64] This long aging forces brewers to wait until the intense mousy off-flavor disappears, costing valuable time and revenue.[22,80] Recent studies showed that in barrel-aged beer, *Dekkera bruxellensis* and *Brettanomyces custersianus* were prevalent in the last stage of the maturation process and even viable after the cleaning procedure of the cask.[61,83] *B. bruxellensis* has the ability to be viable at low oxygen and low pH level.[84,85] However, a recent study suggested mousy off-flavor may mostly be produced by heterolactic bacteria strains *L. hilgardii* and *O. oeni* or chemically in the beverage matrix. Research outcome suggested that *B. bruxellensis* had minimal impact in the development of mousy off-flavor in wines.[14]

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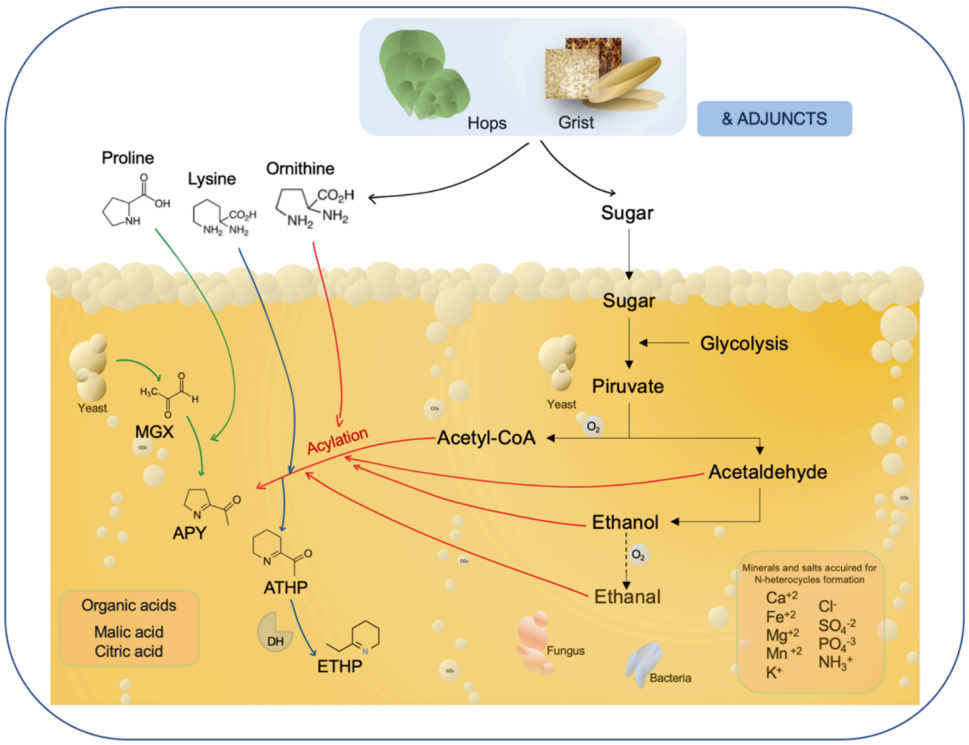

Figure 3. Suggested simplified N-heterocycle formation in a beer matrix. MGX, methylglyoxal; APY, 2-acetyl-1-pyrroline; ATHP, 2-acetyltetrahydropyridine; ETHP, 2-ethyltetrahydropyridine; DH, dehydrogenase.[14−16,56,67]

However, other microbial pathways can be observed, which describe MGX in APY N-heterocycle formation. MGX, when present with proline, can produce APY. MGX is an α-dicarbonyl compound, which is synthesized via *Saccharomyces cerevisiae* during alcoholic fermentation. In the beer matrix, MGX can vary between 2 and 4 μg kg^{-1}.[67,86]

The microbial pathways are suggested for mousy off-flavor development in wine. Although the microflora of sour beers differs from wines, some similarities can be observed, e.g., *B. bruxellensis* and LAB complex during maturation.[7,22] *B. bruxellensis* population is abundant in alcoholic and non/low alcoholic fermented beverages. Although the widest strain variety was obtained in wine samples, specific *Brettanomyces* strains were also found in beer, kombucha, and whiskey beverages, suggesting these beverages can also have an occurrence of N-heterocyclic compounds.[87] In general, LAB and AAB in beer can be found in raw materials, wort, draft dispense, fermentation, packaging, and finished beer and are classified as spoilage bacteria.[88] However, for lambic and ACAs beer types, LAB and AAB are abundant because of the coolship step and cask fermentation. *Acetobacter lambici* and *Pediococcus damnosus* had the highest prevalence during the 30-month lambic-style beer aging. However, over 60 microbial species were identified before fermentation, including colonies of enterobacteria, LAB, AAB, yeasts, and cycloheximide-resistant yeast.[61] Fermentation of the cask consists of four stages: initial, alcoholic fermentation, acidification, and maturation phases. Pyridines, which include ATHP and ETHP, are suggested to be developed at the last maturation phase.[80] In the maturation step, *B. bruxellensis* prevails. However, *Komagataeibacter* in a symbiosis with *B. bruxellensis* is also associated with sensory differences between barrels of the same maturing beer.[89]

Essentially, yeast and bacterial viability depend highly on reducing the sugar content and aerobic/anaerobic conditions. Higher dissolved oxygen and fermentable sugar contents may also be one of the critical factors for THP formation. In particular, the organic acid content is an oxygen-dependent factor in developing mousy off-flavor producing media. However, part of dissolved oxygen is required for yeast and bacteria population viability and odor compounds formation. Before fermentation, the wort can be oxygenated from 5 to 50 mg O_2 kg^{-1}. Wort aeration of 10−12 mg O_2 kg^{-1} resulted in positive outcomes on volatiles and lower acetaldehyde formation, whereas higher concentrations did not significantly impact lowering acetaldehyde.[90] During barrel-aged beer fermentation, the oxygen level decreases. However, one of the cask characteristics is porosity (others are headspace and surface to volume), which allows circulation and initiates oxygen-induced reactions and microbes viability. Also, oxygen can induce ethanal and acetyl-CoA formation, which is needed for N-heterocycles formation (Figure 3).

Higher fermentable sugar content and their chemical structure may increase the unfavorable compound development. A higher concentration of dextrose (glucose), which comes from liquefied adjuncts, such as rice, sorghum, or syrups, may cause the development of off-flavors. Lower levels of monosaccharides can be acceptable, but higher than 5−10 g 100 g^{-1} can interrupt the yeast metabolic pathway, which is responsible for disaccharide maltose fermentation. This current sugar is abundantly released (>55% from fermentable sugars profile) by starch-degrading enzymes during mashing.[12,91]

5. PROTEINS AND AMINO ACIDS IN BEER MATRIX

As discussed above, the amino acid content of lysine, ornithine, and proline in brewing and fermenting matrices is essential for developing N-heterocycles. The grain type of malt and different varieties and growing conditions can influence the levels of these amino acids; for brewing, most of the malt is

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Table 1. Protein and Amino Acid Contents in Barley and Hops

barley origin	protein content (g kg^{-1})	lysine (g kg^{-1})	proline (g kg^{-1})	ornithine (g kg^{-1})	comments	ref
France	91.40−95.6	3.5	9.2−9.4			104
Canada	100.12	3.9	10.2−10.4			
Australia (malted)	47.00−131.10					104,105
Poland (winter varieties)	113.8	2.9	11.4			106
Poland (spring varieties)	131.6	3.4	13.2			
United States (malt)	100.90	4.0	9.0			107
hops	amino acid fraction (g kg^{-1})	lysine (%)	proline (%)	ornithine (%)		
Huell melon variety	22.29	1.1	2.2		measured using HPLC MS/MS	108
Saphir variety	18.66	0.5	5.4			
Hersbrucker variety	14.40	0.7	7.9			
Herkules variety	16.32	0.7	3.6			
Amarillo variety	18.99	0.8	2.2			
	protein content (g kg^{-1})	lysine (%)	proline (%)	ornithine (%)		
aroma varieties[a]	159.20				measured using Kjeldahl method	
bitter varieties[b]	167.00					

[a]Aroma varieties protein content mean using Saaz Saazer, Fuggle, Tettnanger Tettnang, Hallertauer Saphir, Hallertauer Mittelfrüh, Slovenian Styrian Golding, Hallertauer Spalter Select, US Cascade, US Delta, Hallertauer Tradition, Hallertauer Smaragd, Hallertauer Opal, Hallertauer Perle varieties. [b]Bitter varieties protein content mean using Hallertauer Magnum, Hallertauer Nugget, Millenium, Hallertauer Northern Brewer, Super Pride, Hallertauer Herkules, Hallertauer Taurus varieties.

Table 2. Key Flavor and Compounds Responsible for Beer Staling

amino acid	compd	threshold (μg L^{-1})	description	ref
proline	2-acetyl-1-pyrroline	0.053	mousy off-flavor	67
ornithine	2-acetyl-1-pyrroline	0.053	mousy off-flavor	56,82
lysine	2-acetyltetrahydropyridine, 2-ethyltetrahydropyridine		mousy off-flavor	16
β-alanine			wet hay/acetaldehyde, which mirrors tart, green, grassy, cidery or rotten apple like off-flavor	52,115
tryptophan			bitter sweet, sulfitic/sulfidic/corn chip/ethyl butyrate off-flavor	52
valine	2-methylpropanal	86	staling aldehyde, malty aroma	116,117
isoleucine	2-methylbutanal	45	staling aldehyde, malty aroma	116
leucine	3-methylbutanal	56	staling aldehyde, apple-like, "suffocating" flavor, malty aroma	116
phenylalanine	phenylacetaldehyde	105	staling aldehyde, honey-like and grassy flavor	116,118,119
phenylalanine	benzaldehyde	515	almond-like flavor	117
serine, threonine	4,5-dimethyl-3-hydroxy-2(5*H*)-furanone or sotolon	2	Madeira-oxidized−curry−walnut notes, known as madeira off-flavor)	120
methionine	methional	42	at higher concentrations, it is known to be responsible for the light-struck off-flavor of lager beers, staling aldehyde, meaty, boiled potato, onion-like flavor	111,113,121
methionine	dimethyltrisulfide	0.027	onion-like, rotting fruit, sulfury	122
methionine	3-(methylthio)propionaldehyde	250	staling aldehyde (intermediate product of dimethyltrisulfide), cooked potato off-flavor	122−125

from barley. Depending on the barley variety and growing location, lysine content can vary from 0.29 to 0.4% and proline can vary from 0.9 to 1.3% of total protein mass (Table 1). Besides, barley malt can be partly substituted with rice, corn, wheat, buckwheat, oat, and other grains where lysine or ornithine content may vary and influence the development of N-heterocycles.[92−95] According to yeast absorption rate classification, lysine is in group A, and proline is classified to group D. This indicates that lysine is absorbed fast, and proline has a slow absorption rate.[70] Ornithine is an intermediate alkaline amino acid compound (amine) in the biosynthesis of arginine and proline.[96,97] Although ornithine is important for plant growth and stress regulation, scientific data are limited among other grains than rice. Fragrant rice grains have naturally occurring APY, a desirable and key aroma compound.[98,99] In some cases, ornithine is used as a foliar agent to increase APY in aromatic rice growth. After ornithine treatment, APY content varied from 110 to 200 μg kg^{-1}.[100] Higher proline, arginine, or glutamine content may identify higher ornithine content. Few pathways synthesize proline in plants. This amino acid is involved in stress regulation due to heat or drought shock due to the grain fill period. One proline pathway is induced by glutamate in the cytosol and the other by arginine followed by ornithine in the cell mitochondria organelle.[101] Ornithine is an essential precursor for developing abiotic stress-tolerant plants. For example, barley seedling exposure to osmotic stress showed that ornithine increased foliar proline content.[102,103]

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Hops are an essential raw material in beer production for flavor and harmonized quality assurance. It also contains proteins, which can be released during brewing and fermentation processes, especially with an increased dry-hopping procedure. Crude protein content in hops varies from 13 to 20%, from which the amino acid fraction comes to 10%.[94,95] Depending on the variety, the lysine content may vary. Centennial hops have approximately 0.5% (of total mass) of lysine.[110] Huell Mellon variety had 1.1%, and Spahir, Hersbrucker, Hercules, and Amarillo varied between 0.5−0.8%. Proline content varied from 2.2 to 7.9%.[108] Once again, biochemical composition proves that the raw material selection for beer brewing is important for developing a balanced flavor beverage. Hops are used to add bitterness and aroma to beer. In lightly bittered beer styles, hops were added during the boiling stage in brewing. But most of the hop aroma is driven off during the boiling process. To further enhance the aroma, a process of dry hopping, or adding hops into the fermenter during fermentation, or to add hops immediately after fermentation is used.[122] For each of these additions of hops, more amino acids would be added.[110] However, bitter compounds from hops like iso-α-acids and their derivatives like humulinone interact with LTP and protein Z, resulting in complexes which positively impact foam stability and lacing. Humulinone has several sites from which can bind to protein Z, including Asn-37, Ser-292, Lys-290, and Pro-395, and it can be introduced to beer through dry hopping, which results in a decrease in soluble protein content or free amino nitrogen content.[111,112] However, free amino nitrogen content measured in sweet, hopped, and unhopped wort suggested that nitrogen increases after the hopping procedure.[113] In general, the proteomics of hops is partly revealed.[114] Even so, targeted experimental studies on hop proteins in beer to investigate mousy off-flavor development and mitigation strategies are needed. Hence, understanding the total amino acids and specifically the amino acid profile would help brewers understand the risks of amino acid derived off-flavors. Examples of amino acid involvement in staling beer compounds are presented in Table 2.

6. DETECTION AND PREVENTION OF N-HETEROCYCLES

The beverage matrix and nature of the compositional structure play a key role in THP and APY identification using gas or liquid chromatography instruments. Mousy off-flavor compounds are odorants. However, their protonation, isomerization, and intensity highly depend on if the medium is alkaline or acidic. The identification of a mousy off-flavor has been proposed in a wine matrix. Although the pH of testing samples is between 3.5 and 4, clean samples before injection are highly alkylated to 9−9.5. This step produces a similar chemical structure of the N-heterocycles. Kiyomichi et al. (2023) limits of detection (LOD) of ATHP, ETHP, and APY for white, rose, and red wines using stir bar sorptive extraction gas chromatography−tandem with mass spectrophotometry (SBSE-GC-MS) were 0.5−0.8 μL kg^{-1} for APY, 0.8−10.7 μL kg^{-1} for ATHP, and 0.6−2.7 μL kg^{-1} for ETHP. Limits of quantification (LOQ) were 1.8−3.1 μL kg^{-1} for APY, 2.6−36.0 μL kg^{-1} for ATHP, and 2.0−8.9 μL kg^{-1} for ETHP. In comparison, research conducted by Hayasaka (2019) using liquid chromatography−mass spectrophotometry utilized atmospheric pressure ionization (HPLC-APCI-MS) for ATHP identified LOD and LOQ were ten and more times lower.[55,62] However, LC/MS method did not include ETHP and APY detection, and they are associated with the presence of ATHP and ETHP.[55] Also, APY identification in grain, specifically fragrant rice, using a simultaneous distillation−extraction method (SDE) following GC/MS, has been reported.[68] Other study with roasted barley tea successfully isolated odor compounds profile including APY in variation of 0.82−1.02 μL kg^{-1}. Nonvolatile compounds from the hydrophilic extract were removed using solvent extraction and solvent-assisted flavor evaporation (SAFE).[126] Unfortunately, there are limited studies and many knowledge gaps in THP, APY, and related compound identification for beer samples. Suggested methods with modifications may be applicable for the detection. Both beer and wine production involve extractions, maceration, and mashing, which releases bioactive compounds, minerals, and vitamins. Wine is rich in phenolic compounds such as anthocyanins and tannins and volatile flavor compounds.[127] Beer involves a slightly more complex matrix due to Maillard and Strecker reactions, and micro and macro components from grains and hops. There are over a few hundred acids, carbonyl compounds, and over 600 flavor compounds.[128] Complex and style-dependent beer matrix as well as highly pH-based stability on N-heterocycles present a challenge for reliable method development using liquid or gas chromatography.

In general, non/low alcoholic and alcoholic beer production can influence N-heterocycles production. Selection of low colored malt with a lower kilning heat load of which Maillard and Strecker reaction degrading products, such as ATHP and APY, can increase by reducing boiling time, where some heat-induced reactions occur. Also, the biochemical compositions of selected malt and hops can significantly improve the end products. Higher lysine, ornithine, proline, iron, calcium, and magnesium content may impact the fermentation process of selective and spontaneous microbial strains, which initiates the catabolism of mousy-off flavor compounds.[56] However, different technologies can produce sour beers: spontaneous cask fermentation, pitching LAB, and creating cofermentation with yeasts or other microbes. To minimize their spontaneous occurrence, it was suggested incorporating heterolactive fermentation-initiating yeast strains, e.g., *Hanseniaspora vineae*, *Lachancea fermentati*, *Lachancea thermotolerans*, *Schizosaccharomyces japonicus*, and *Wickerhamomyces anomalus*.[129] To improve kettle sour beer production by calculating pitching LAB bacteria likewise *Lactobacillus casei*, *Lactobacillus helveticus*, and *Lactobacillus rhamnosus* Cyllometer X2 image cytometer may provide an efficient bacterial count to minimize timing and ensure consistent and quality products.[130] This novel imaging tool that implements fluorescence can also be applicable for standardizing other spontaneous and mixed-culture beer fermentations. However, nonpitched probiotic *Lactobacilli* sp. cannot survive in hoppy beer due to high antimicrobial activity. Some modifications during fermentation can be implemented to maintain viability.[37,131]

Beer is one of the most consumed beverages in the world, and a balanced flavor profile is essential for the best consumer experience. Mousy off-flavor is a common challenge in sour beer types. The current literature review identifies that chemical and microbial pathways may be responsible for mousy off-flavor development in beer matrices. Due to the complex beer brewing process and overall supply chain, evaluating each pathway's importance level is challenging. However, observations identify that mousy off-flavor develop-

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ment consists of interconnected parts, which key elements include microbial diversity and viability, sugar, and dissolved oxygen content, and specific amino acid's role. Lysine, ornithine, and proline amino acids play important precursor roles in the development of THP and APY.

Beer produced from darker malts where Maillard and Strecker degradation products are involved in complex pathways during fermentation may increase mousy-off flavor abundance. Despite these observations, pursuing the methods to identify and capture the formation of ATHP, ETHP, and APY in the beer matrix is essential to evaluate dynamics and prevent off-flavor development. An uncontrolled process can lead to high economic and time-consuming losses. By understanding the biochemical pathways of N-heterocycle development, mousiness can be reduced to an acceptable and desirable amount by selecting raw materials, controlling heat-loaded procedures, and observing fermentation metabolomics such as individual amino acids.

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Notes

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ABBREVIATIONS

AAC, acetic acid bacteria; ACA, American coolship ale; APY, 2-acetyl-1-pyrroline (imine form) and 2-acetyl-2-pyrroline (enamine form); ATHP, 2-acetyl-1,4,5,6-tetrahydropyridine (enamine form) and 2-acetyl-3,4,5,6-tetrahydropyridine (imine form); EBC, European Brewery Convention; ETHP, 2-ethyl-1,3,4,5,6-tetrahydropyridine (enamine form) and 2-ethyl-3,4,5,6-tetrahydropyridine (imine form); FTHP, 2-formyl-1,4,5,6-tetrahydropyridine; GC-FTIR, gas chromatography−Fourier transform infrared spectroscopy; GC-O, gas chromatography−oflactometry; GC/MS, gas chromatography/mass spectrometer; IoB, Institute of Brewing; LAB, lactic acid bacteria; LC/MS, liquid chromatography/mass spectrometer; LS, lambic-style; MGX, methylglyoxal; NABLAB, non/low alcohol beverages; THP, tetrahydropyridine; 2M3MB, 2-mercapto-3-methyl-1-butanol; 3M3MB, 3-mercapto-3-methyl-1-butanol

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