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Original Article

Differentiating Pathogenic Bacteria through Biochemical Markers: A Study for Clinical Applications

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ABSTRACT

Introduction: Beer has become the world's largest consumed alcohol. Beer brewed on a small scale in terms of quantity by independent breweries that brew traditionally has been termed as craft beer. Small-scale breweries involve testing newer preparation techniques, which also involve the use of raw materials not commonly accepted. However, these practices can increase the incidence of contaminants, awareness of which helps consumers make informed choices. Contamination can occur at any stage of the production process, and microbial contaminants may either improve or deteriorate the quality of beer by producing biochemicals and organic acids, affecting the beer's shelf life.

Aim: To analyse various local craft beer samples for contaminants, with a focus on microbial contamination.

Methods: Samples of the local craft beers were taken and screened. The samples were diluted serially and spread plated on simple agar media for isolation. Gram staining and biochemical characterisation of bacterial colonies

Results: There are several kinds of microorganisms identified to exist in local craft beers. Their existence is detected through the characteristic and biochemical profiling in the samples.

Conclusion: The study indicates the presence of microbial contamination in local craft beer, thereby showing the possible effects of such contamination on quality and shelf life. Consumers and producers could use this information to guide decisions to produce and consume craft beers.

Keywords: Alcoholic beverage, Craft beer, Microbial contamination, Spoilage

BACKGROUND

Beer is one of the beverages in the utmost demand among consumers, besides water and tea. It was discovered only in the late nineteenth century that yeast converts sugar into ethanol or ethyl alcohol and carbon dioxide during the process known as fermentation [1]. This paper tries to analyse craft beer, the kind of beer that has gained popularity within the minds of young consumers in the world, by increasing the number of craft breweries to 17,000. A craft brewery is a brewer that produces beer on a small scale, rich in traditional ingredients, for their historic styles, and with the addition of non-traditional ingredients that enhance the uniqueness of the beer. Craft brewers, their beer, and their industry are independent of the other alcoholic sectors. Raw materials, alcohol content, age of the beer, and packaging are what make craft beer unique. Since craft beers are not usually pasteurised, though rich in health compounds, they have a reduced shelf life [2]. Any stage during the production process can prove to be the source of contamination. These can be of a physical, chemical, and biological nature, just exactly of a microbiological nature. Such microbiological contaminants add value or degrade the quality and shelf life of craft beer.

The industrial brewery has many automated systems installed for measuring and the maintenance of parameters like temperature, pH, oxygen, turbidity, foam, and other control systems for the production of alcohol. In contrast, the conventional craft beer usually has reduced funding and does not employ such control systems despite their essentiality, which paves several other production, safety, and quality problems in the brewery [3]. Beer has certain antimicrobial properties, like high carbon dioxide, low oxygen, alcohol content, and low pH, which prevent spoilage by microorganisms, especially food pathogens. However, these have limited control over the spoilage microorganisms, and these can potentially spoil the texture, flavour, colour, as well as the taste of the consumable beer and make it unfit for human consumption [4].

The craft beer has highly distinguishable extrinsic and intrinsic characteristics [5,6]. The extrinsic characters include the brand, packaging, labelling, and the country of origin, and the inherent characters include the colour, flavour, taste, smell, and the alcohol content. It is of significance to note that the craft beers are produced by a complex combination of the raw materials of the traditional beer like malted and unmalted cereals like barley, wheat etc., hops, and yeast with the non-traditional ones (fruits, flowers, spices, etc.), in varied proportions and by using the customary and the newer brewing techniques, which results in the wider range of beer styles and types [5,6].

Beer mainly consists of water, malt, hops, and yeast. Some styles have aromas and flavours coming from the fruits, flowers, and spices that are used as raw materials that have been acted upon by yeast. Alcohol content or alcohol by volume of beer is the measure of the alcohol contained in a given volume of beer. The ABV of an ordinary beer can go from 3 to 14%, but the most popular ones must not exceed 6% [7].

This results in physical, chemical, and microbiological contaminants of the beer, which implies a great effect on health. In most cases, recently developed flavours and styles are those that are cherished in most craft beers. Several source materials, unhygienic procedures of production, poor pasteurisation processes, unfriendly industrial environments, or little alcohol content during the beer fermentation process, have all been identified as possible sources of contaminants [8].

Microbiological impurities can be primary and secondary depending on the time at which it is contributed to the beer. Those impurities that are offered through the raw materials form the primary impurities; the secondary impurities are those that are introduced at the finished product [9,10]. Lactobacillus, Pediococcus, Staphylococcus, and yeasts and fungi like Saccharomyces, Rhodotorula, Alternaria, Hansenia, Wickerhamomyces, and Cladosporium comprise some of the spoilage and non-spoilage microorganisms that can be implicated in the craft beer [11].

MATERIALS AND METHODS

Three craft beer samples were analysed from a local microbrewery. To isolate the bacteria from the samples, Nutrient Agar (NA) Media was used.

The identification of bacteria was done by performing the Gram staining reaction and a series of biochemical tests.

Objectives

1. Isolation of bacteria from different samples of craft beer.
2. Identification of the isolated bacteria.

Isolation of the bacteria

This study is based on a local microbrewery that hosts different styles of craft beers and alcohol. Three samples

of craft beer – Bira Blonde Lager, Simba Stout, and Toit Weiss were collected. The collected samples were serially diluted and cultured on Nutrient Agar plates. The culture plates were incubated for 18- 24 hours at 37 0C in the incubator.

Identification of the bacteria

Gram staining reaction was performed on all sixteen isolated bacterial colonies. Seven biochemical tests were carried out on the isolated bacterial colonies to help identify them.

Gram Staining Reaction

1. Gram-positive organisms appear purplish-violet in colour

2. Gram-negative organisms appear pinkish-red in colour.

Catalase Test

1. Positive test results in the production of effervescence or bubbles when H2O2 is added.

2. Negative test results in no bubble formation.

Oxidase Test

1. Development of a deep purple colour after the addition of Kovac's reagent indicates a positive oxidase test.

2. A negative test gives no colour change.

Simmons Citrate Test

1. Citrate utilisation by the bacteria produces an alkaline reaction on the agar slant and changes the colour of the medium from green to blue.

2. A negative test renders no change in the colour, i.e., the medium remains green after the incubation.

Indole Test

1. Formation of a pink to red colored ring in the broth culture upon addition of Kovac's reagent indicates a positive test for indole.

2. No colour change indicates a negative test.

Methyl Red Test

1. A distinct red colour formation after the addition of methyl red dye to the incubated broth tube indicates a positive test.

2. A negative test is indicated by no colour change after the addition of the dye, i.e., the medium remains pale yellow in colour.

Voges Proskauer Test

1. A pinkish-red colour at the surface of the tube after the addition of Barritt's Reagent A and Reagent B indicates a positive test for VP.

2. No colour change in the tube after the addition of the respective reagents indicates a negative test for VP.

Urease Test

1. Appearance of bright pink colour after the incubation of the tubes indicates a positive urease test.

2. No colour change in the medium indicates a negative test for urease

RESULTS

Research conducted on microbial contamination in local craft beers uncovered important details about the various bacteria found in the samples. The craft beers Bira Blonde Lager, Simba Stout, and Toit Weiss underwent isolation and identification of contaminants using the Gram Stain and various biochemical assays.

The samples revealed a diverse range of bacterial colonies. Some of the colonie colours were opaque, creamy, or frothy, while others were a brilliant, lumpy yellow. Many of the colonies were round and raised, with a slimy, pebbly feel, and the same moisture levels were observed on the colonies. Table 1 shows the variation in colony morphology of the bacterial colonies that have been isolated. The Gram stain results of the colonies were positive or negative for Gram, thus accounting for the presence of Bacillus subtilis, Pediococcus acidilactici, Listeria monocytogenes, Lactobacillus sp., Staphylococcus aureus, and some Klebsiella sp. The Gram-positive and negative reaction results of these expectable representatives are visible in Figure 1, while some of the isolates are Bacillus subtilis, Pediococcus acidilactici, and Listeria monocytogenes.

In order to gain some insight into the distinct biochemical patterns and phenotypes of the isolated organisms, the following biochemical tests were performed: For Bacillus subtilis, Bacillus cereus, and Lactobacillus delbrueckii, these organisms were identified as being catalase positive, and for Pediococcus acidilactici, it was identified as being urease positive. As for the remaining genus, which includes Staphylococcus aureus and Klebsiella pneumoniae, these species were identified as being oxidase-negative. The biochemical tests performed on the isolates are summarised in Table 2, which illustrates the results associated with various biochemical tests, i.e., positive and negative catalase, oxidase, citrate, indole,

methyl red, Voges-Proskauer, and urease, as well as their biochemical test results.

The results indicate that the Bacillus species that include Bacillus subtilis were the ones that tested positive for the Voges-Proskauer test, which demonstrates their ability to ferment carbohydrates and produce an acetyl methyl carbohydrate. In addition, it was also observed that some other species, which include Pediococcus acidilactici, also tested positive for methyl red, which indicates that they produced an acid during the process of fermentation.

This research detected and characterised a variety of bacterial taxa, each exhibiting distinct biochemical activities. Bacteria of this type might change the taste, mouthfeel, pH, and scent of craft beer, leading to its spoilage. The research reveals a concerning vulnerability to microbial spoilage of craft beers, and particularly the unpasteurized products. This underscores the need for effective sanitation in small-scale brewing operations to protect the integrity of the product. The next phase of this research will employ molecular methods, including PCR, to characterise these microorganisms and explore their potential consequences on the safety and quality of craft beer.

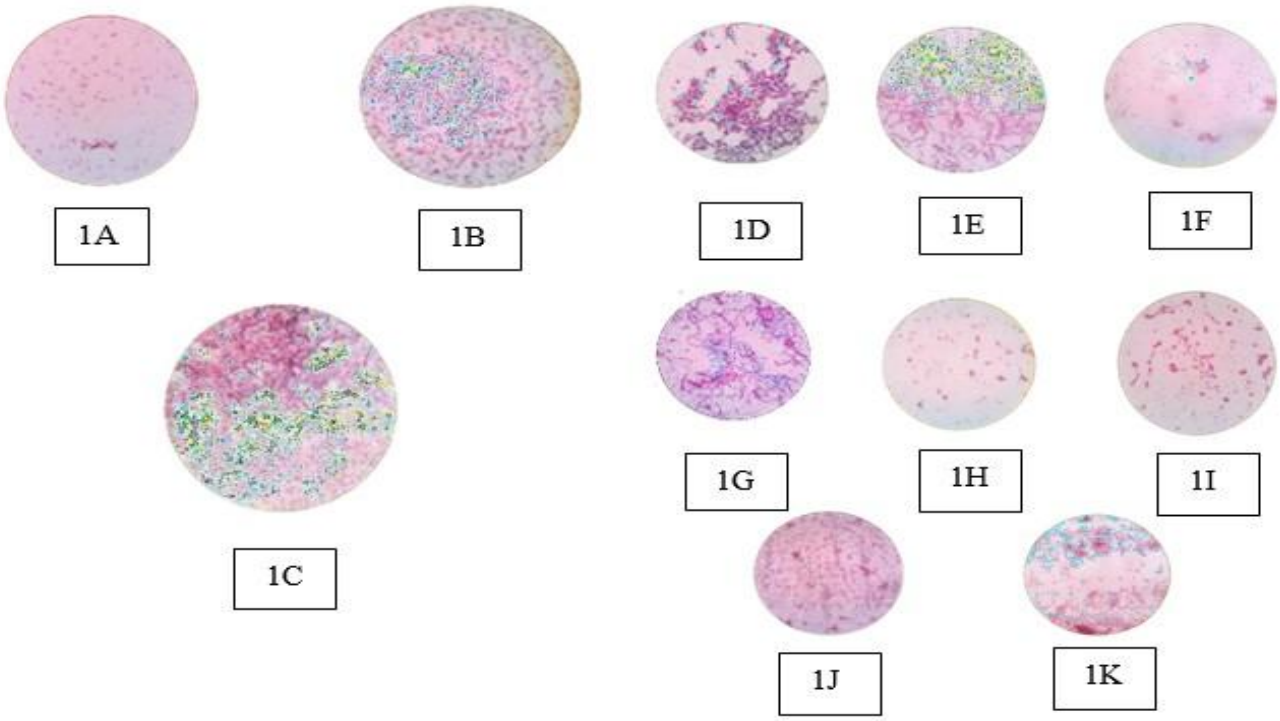

Figure 1: Gram staining reaction of the isolated bacteria

(**1A**: Bacillus subtilis, **1B:** Pediococcus acidilactici, **1C:** Listeria monocytogenes**, 1D:** Lactobacillus sp., **1E:** Lactobacillus sp., **1F:** Bacillus aeolius, **1G:** Lactobacillus sp., **1H:** Klebsiella sp., **1I:** Staphylococcus aureus, **1J:** Acetobacter sp., **1K:** Enterobacter sp.)

Table 1: Colony Morphology of the isolated bacterial colonies

Sl. No.	Margin	Color	Elevation	Texture	Shape	Gram Reaction
1.	Lobate	Yellow	Raised	Slimy, moist	Round	+
2.	Lobate	Opaque/white	Flat	Slimy, moist	Round	+
3.	Undulate	Opaque/white	Raised	Slimy, moist	Round	+
4.	Entire	Opaque/white	Convex	Smooth	Round	+
5.	Entire	Opaque	Raised	Smooth	Round	+
6.	Undulate	Opaque/white	Convex	Slimy, moist	Round	+
7.	Entire	Orange	Raised	Smooth	Round	+
8.	Undulate	White	Raised	Shiny, viscous	Round	-
9.	Entire	Translucent	Raised	Smooth	Round	-
10.	Irregular	Opaque	Raised	Smooth	Round	-
11.	Entire	Opaque/white	Umbonate	Smooth	Round	-
12.	Entire	Opaque	Raised	Slimy, moist	Round	-
13.	Entire	Opaque	Raised	Slimy, moist	Round	-

Table 2: Bacterial Characteristics

Characteristic	B. subtilis	B. cereus	B. aeolius	L. delbrueckii	S. aureus	S. xylosus	S. roseus	K. pneumoniae	P. acidilactici
Gram reaction	+	+	+	+	+	+	-	-	+
Catalase	+	+	-	-	+	+	+	+	-
Oxidase	+	+	+	-	-	-	+	-	-
Citrate	+	+	+	-	+	+	+	+	+
Indole	-	-	+	-	-	-	-	+	+
MR (Methyl Red)	-	+	+	-	+	+	-	-	-
VP (Voges Proskauer)	+	+	+	-	-	+	-	+	-
Urease	-	+	-	-	+	-	-	+	-

DISCUSSION

It was only during the course of the nineteenth century that it was discovered that yeast converts sugars into alcohol in the process of fermentation. Though the ancients did not have the necessary idea about these compounds and organisms, they used this process to make some intoxicating beverages. The ancient Europeans made fruit-based, honey-based, and barley-based beverages known as wine, mead, and beer, respectively. No matter how many other substances are added during preparation, anything made from maltose is beer. Currently, commercial beers are usually brewed using malted barley, often with added malted or unmalted other grains like wheat and rye, as well as hops [10-12].

Beer is the most widely consumed and tolerated alcoholic drink globally. Independent craft breweries have sprouted in tremendous numbers worldwide over the past two decades. Craft beer producers design new and novel ingredients that alter the taste of beer, colour, scent, and flavour. Industrial beer and craft beer are susceptible to microbial contamination as they contain all the growth factors and nutrients [13].

Craft beers are normally brewed in smaller breweries that are not like the commercial brands, brewed by giant industrial breweries. The differences come into the taste and contents used, but also in the way it is made. While large-scale beer is often not pasteurised or sterile-filtered in the final steps of production, it is more susceptible to microbial development [14]. The contaminants in the beer can be identified using the various selective and differential media; however, they are relatively time-consuming compared to modern molecular techniques. Due to the combination of molecular techniques and the microbiological assessment, the contaminants in beers can be identified accurately. So, good sanitation in these microbreweries is considered highly important to avoid these unfavourable organisms in the product. Currently, many breweries use a cleaning-in-place method to test the keeping quality of the beer [15]. This system is in place not only to avoid and reduce the risk of contamination, but its existence also prevents economic loss to manufacturers. The more common beer-spoilage bacteria are Gram-positive lactic acid bacteria and Gram-negative acetic acid bacteria. Sometimes, bacterial spoilage and contamination of beer might occur, and suspects may include Staphylococcus, Bacillus, Enterobacter, and Zymomonas. This depends on the kind of microorganism, but may affect pH, sedimentation, texture, ropiness, and off-flavour [16,17]. The species of bacteria are Lactobacillus brevis, L. lindneri, L. paracasei, L. plantarum, and some species of Leuconostoc, which are generally normal contaminants of craft breweries [12-17]. Wild yeasts produce off-flavours, different content of alcohol, changes in turbidity and carbonation, and loss of beer during processing [18].

Different microbiological and culturing methods have been conventionally studied to identify contaminants in beers. However, the recent development in molecular techniques such as PCR, chemiluminescence, bioluminescence, mass spectroscopy, and flow cytometry has made it possible to do a proper assessment of the beer [19].

LIMITATIONS

There are multiple limitations when it comes to studying microbial contamination of local craft beers. To begin with, the research had a very limited scope, sampling only three styles of craft beer from a small microbrewery. Typically, the craft beer sector is considerably larger, and that makes it hard to extrapolate the study's conclusions to other craft beers and breweries. Moreover, the research study in question utilised traditional microbiological practices, notably Gram staining and various biochemical procedures. Even though these approaches are useful, they consume a lot of time, and comparatively, the accuracy of other contemporary practices is better, including molecular techniques like PCR. It is also reasonable to criticise the research for concentrating just on bacterial contamination, as it did not consider other forms of contamination, notably fungal, which could have contributed to the spoilage of the beer. Also, the study confirmed the existence of microbial contaminant organisms but failed to determine their potential impact on the beer's sensory and safety attributes. Moreover, this is the most important dimension that needs to be fully researched in order to understand the complete impact of these microorganisms. Also, the study did not control for the differences in the level of contamination for various production batches. This is important, as it could lead to differences in the results of the study.

FUTURE AIMS AND SCOPE

This study also aims to evaluate microbial contamination of craft beers through the use of a molecular approach, Polymerase Chain Reaction (PCR), which will allow for better precision in the identification and characterisation of microorganisms. With this approach, the identification and understanding of the specific organisms and species will be more precise than traditional microbiological methods. Furthermore, the research aims to expand the study by involving a more extensive selection of craft beers from various breweries to see if the results are similar with different craft beers and different batches.

Additionally, the study aims to address potential fungal contamination in craft beers since the current study only

focused on bacterial pathogens. Future research will focus on the identification of fungal species implicated in beer spoilage. The impact on the sensory characteristics of the beer, including flavour, aroma, mouthfeel, texture, and overall quality, will be determined to clarify the consequences of microbial contamination on the consumer experience.

The purpose of the research is to study the impact of specific microbial contaminants on the safety and shelf life of craft beer, particularly how this changes the health risk paradigm of the beverage. This will be achieved through a combination of laboratory analysis and consumer studies to assess the overall impact of microbial contamination on producers and consumers. The primary objective is to enhance the quality control procedures in craft beer and to establish guidelines on microbial contamination for small-scale breweries.

CONCLUSION

The above study helped us evaluate the various contaminating and non-contaminating microorganisms present in the beer sample. With the Gram staining reactions and the various biochemical tests performed, the isolated organisms were identified. In the subsequent phases, we aim to delve deeper and identify these microorganisms using polymerase chain reaction (PCR) and culture the bacteria in various enriched, selective, and differential media used for the isolation of bacteria from beer samples, like Universal Beer Agar (UBA), Lee's Multi-differential Media, Wallerstein Nutrient Agar (WLN), Raka-Ray Lactic acid bacteria (RRLM), MacConkey agar, etc. This study involves the isolation and identification of only bacteria, and the next phase of the research aims to isolate and identify fungi from the samples. We also aim to investigate a vast multitude of samples that can provide a broader perspective for the said evaluation. In light of the above findings, our future work also focuses on determining the positive and negative implications of these isolated bacteria on the quality and health of the craft beer.

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AUTHOR CONTRIBUTIONS:

Anusha Ravi: Conceptualized the study, developed the research design, and oversaw the overall project. He was responsible for data analysis and interpretation, as well as drafting the manuscript.

Swati Chandrakant Hosvakka: Assisted in the design and implementation of the study. Contributed to the development of the survey instrument, data collection, and analysis. Provided critical revisions to the manuscript.

Sumana Kumar: Participated in the literature review, contributed to the data collection process, and assisted with the writing and editing of the manuscript. She also helped interpret the findings and offered critical feedback on the manuscript.

ABBREVIATIONS USED IN THE STUDY:

a) **NA** - Nutrient Agar
b) **MR** - Methyl Red
c) **VP** - Voges Proskauer
d) **PCR** - Polymerase Chain Reaction
e) **UBA** - Universal Beer Agar
f) **WLN** - Wallerstein Nutrient Agar
g) **RRLM** - Raka-Ray Lactic Acid Bacteria Medium

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