DEPARTMENT OF AGRICULTURAL, FOOD AND ENVIRONMENTAL SCIENCES

DEGREE COURSE: FOOD AND BEVERAGE INNOVATION AND MANAGEMENT

CHARACTERIZATION OF LACTIC ACID BACTERIA FROM A KVASS-LIKE BEVERAGE PRODUCED USING RYE (*SECALE MONTANUM*) FLOUR

TYPE OF DISSERTATION: Research

Student:
NOEMI ANZINI

Supervisor:
PROF. ANDREA OSIMANI

Assistant supervisors:
PROF. JOANNA HARASYM

DR. GIORGIA RAMPANTI

ACCADEMIC YEAR 2023-2024

To those who supported me with trust and to those who, not believing in me, gave me the strength to overcome my limits and achieve this milestone, I dedicate this thesis.

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ACRONYMS AND ABBREVIATIONS

AW	Water Activity
AX	Arabinoxylans
EA	Emulsifying Ability
ES	Emulsion Stability
FC	Foaming Capacity
FS	Foaming Stability
FFCBs	Fermented Functional Cereal Beverages
HePSs	Heteropolysaccharides
HoPSs	Homopolysaccharides
HLI	Hydrophilic/Lipophilic Index
LGC	Least Gelation Concentration
NAFB	Non-Alcoholic Fermented Beverage
RVA	Rapid Visco Analyzer
SRPE	Sterile Rye Bread Extract
SP	Swelling Power
PA	Phenolic Acids TPA
TPA	Texture Profile Analyses
OAC	Oil Absorption Capacity
WAC	Water Absorption Capacity
WAI	Water Absorption Index
WHC	Water Holding Capacity
WSI	Water Solubility Index

INTRODUCTION AND AIM OF THE THESIS

Kvass is a fermented cereal beverage traditionally produced from rye flour and stale rye bread, widely consumed in Eastern European countries (Dlusskaya et al., 2008). Kvass production exemplifies how fermentation can help reduce food waste by transforming a waste product (stale bread) into a new product (kvass). Moreover, kvass represents an alternative for individuals with milk protein allergies, as well as for those who prefer non-alcoholic beverages or cannot consume alcohol, such as pregnant women and children. Additionally, in non-Eastern European countries, kvass could appeal to people interested in trying new products, particularly functional foods, due to its concentration of bioactive compounds that offer potential health benefits.

However, authentic kvass, produced using traditional methods, has almost completely disappeared from the market. Instead, what is labeled as kvass today is often a diluted grain extract concentrate mixed with water, colorings, artificial flavors, and sweeteners, and containing a high amount of preservatives (Klosse, 2013). This shift can be attributed to industrial needs for shelf stability, repeatability, and uniform production, which are easier to achieve with these modified formulations.

In this study, kvass was produced at laboratory-scale through the spontaneous fermentation of a mash made from homemade wholemeal rye bread, prepared using traditional methods. The aim of the study was to isolate lactic acid bacteria from the end product and select potential starters for industrial applications.

CHAPTER 1

1.1 Cereal-based fermented beverages

Food and beverages prepared though fermentation represent an important part of human nutrition and hold significant cultural importance worldwide. Since early human history, gathering edible ingredients has been essential for survival and maintaining physiological functions. In this context, fermentation is one of the oldest methods of food preservation, second only to drying, with origins dating back to ancient times. Over centuries, fermentation has evolved alongside society, contributing to a diverse array of food products that reflect different cultures.

The choice of the raw materials for fermentation is influenced by factors such as ethnic preference, societal pattern, religions, climate, and topography. As a process, fermentation involves the transformation of simple raw materials int value-added products through the growth of microorganisms and their metabolic activities on different substrates. This process not only plays a crucial role in enhancing food security, but also improves the nutrient composition by transforming the raw materials into products with different sensorial properties and simultaneously reducing their antinutritional effects (Tamang & Kailasapathy, 2010). Fermented food and beverages, as noted by Tamang et al. (2010), make up a significant part of traditional diets in many parts of the world, underscoring their widespread diffusion and consumption. This demonstrates how these beverages are fundamental in many cultures for their nutritional properties and health benefits. However, despite their historical and nutritional importance, the consumption of certain lesser-known and traditional fermented foods is declining. Several factors contribute to this trend, including changes in lifestyle, a shift towards commercial foodstuffs and fast foods, and in some regions, the impact of climate change, which affects traditional culinary practices (Farnworth, 2003).

Consumer lifestyles are constantly evolving due to globalization. Over the past few decades, human health and food safety have gained increasing importance due to the established link between nutrition and the development of various health problems. The presence of bioactive compounds in food can play an important role il prevention of illnesses (Ignat et al., 2020).

Among food products, “functional foods” are able to provide bioactive compounds and essential nutrients to the human body. Functional beverages, in particular, offer an effective way to deliver these nutrients and bioactive compounds, enhancing their absorption and bioavailability. These beverages encompass various categories such as dairy-based, fruit and vegetable-based, legume-based, cereal-based, coffee, and tea.

A popular category of fermented beverages includes those made from cereals such as barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), millet (*Panicum miliaceum* L.), oats (*Avena sativa* L.), rice (*Oryza glaberrima*/*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*), and wheat (*Triticum aestivum* L.) (Blandino et al., 2003). These cereals are good fermentation substrates and also possess potential functional properties, as they contain nutrients readily assimilated by probiotics (Valduga et al., 2019). Cereals belong to the grass family, Gramineae. Wheat, rice, maize, rye, oats, barley, sorghum, and millet are grown in high quantities in different countries. They constitute a significant portion of daily energy requirement, thus are considered as “staple food”. The leaders in the cereal production are USA, India and China countries. Apart from providing the daily calorific intake, cereals are grown and consumed all over the world for their nutritional properties and benefits. Cereals and their products are principal source of energy, carbohydrates, dietary protein, fiber, minerals, vitamin E and some of the B vitamins. They also have a long storage life due to low moisture content of the grains.

Most traditional and currently produced non-alcoholic fermented cereal beverages (NFCBs) are regarded as functional foods and wholesome nutritional products (Yu & Bogue, 2013).

Consumption of these beverages has the potential to reduce the adverse health and economic impacts of poor diets. Additionally, a significant benefit of cereal-based beverages is their suitability for consumption by vegetarians, vegans, and lactose-intolerant consumers (Menezes et al., 2018).

Currently, “non-alcoholic” is a regulatory term, and its definition varies by country. For instance, in European Union, regulation no. 1169/2011 states that a beverage must be labelled as alcoholic if it contains ethanol by volume of over 1.2% (Ignat et al., 2020).

1.2 **Kvass classification and production**

Kvass is a cereal-based fermented beverage traditionally produced from rye and barley malt, rye flour, and stale rye bread, and is mainly consumed in Eastern Europe, especially in Russia. Classified as non-alcoholic fermented beverage (NAFB), it typically has an alcoholic content not exceeding 1.2% (Baschali et al., 2017).

As shown in Figure 1, two primary methods are employed in the production of kvass, utilizing either stale sourdough bread or malt as the primary ingredients (Dlusskaya et al., 2008). In both cases, the natural microbial component of rye is responsible for the grain's fermentation. In the fermentation process using stale sourdough bread, all necessary sugars for yeast fermentation are derived from the bread-making process, so no additional sugars are needed. Alternatively, in the second technique, gelatinized starch is broken down by malt enzymes. Prior to fermentation, the kvass mixture is diluted in boiling water and clarified through sedimentation. Sucrose is then introduced to the kvass wort, and fermentation is initiated by adding baker's yeast or a previous batch of kvass. Fermentation is stopped once the product is chilled to 4°C before nutrient depletion. In contrast to sourdough bread and similar fermented cereal products, kvass undergoes no post-fermentation heat treatment, thus maintaining high cell counts of viable yeast and lactic acid bacteria. When made at home, a sourdough stock culture is used as a starter for the fermentation. At industrial level, kvass is produced using starters, and the final product is often pasteurized and supplemented with preservatives (Baschali et al., 2017).

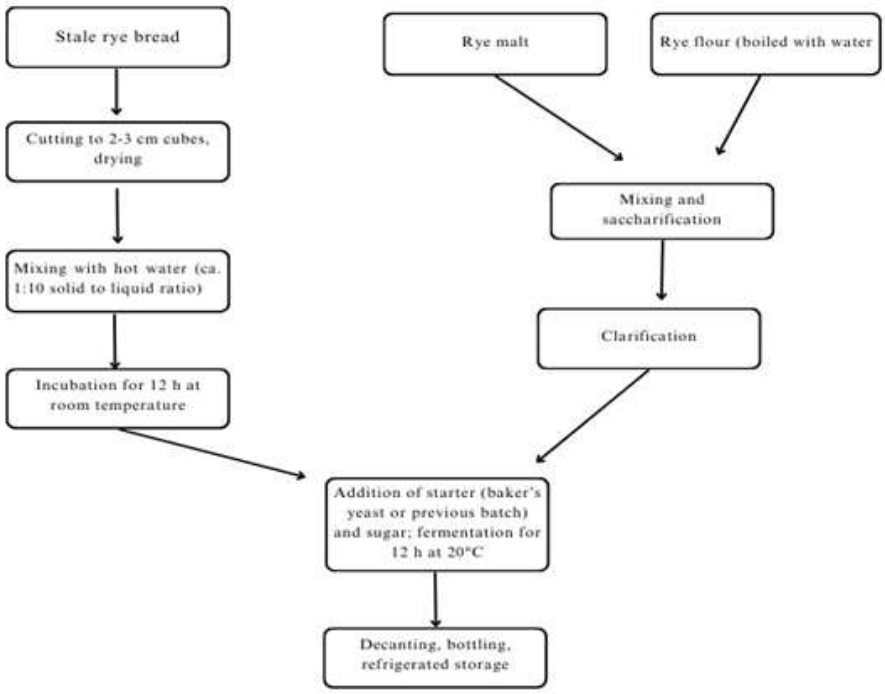

Figure 1: Flow chart showing the production methods of kvass.

1.2.1 *Microorganisms involved in kvass production*

Artisanal starters maintained through continuous back-slopping remain the most common method to initiate kvass fermentations (Dlusskaya et al., 2008). These starters not only enrich the biological diversity of traditional kvass but also promote its development as a live-bacteria beverage.

The species-level composition in kvass varies widely due to differences in fermentation techniques and raw materials. Despite this variability, lactic acid bacteria (LAB) and yeasts are the predominant microorganisms in the kvass fermentation process. The literature indicates a symbiotic relationship between these two groups of microorganisms (Ramos et al., 2011). Indeed, during fermentation, LAB create an acidic environment optimal for yeasts growth, while yeasts produce amino acids and vitamins that enhance bacterial growth. *Saccharomyces cerevisiae*, the main yeast specie, together with LAB, imparts the characteristic taste and aroma of kvass. The resulting low pH from fermentation acts as a natural preservative, inhibiting the growth of undesirable microorganisms (Alu'datt et al., 2019; Gänzle & Salovaara, 2019).

In literature, prevalent LAB species identified in kvass include *Leuconostoc mesenteroides* (Lidums & Karklina, 2014)*, Lacticaseibacillus paracasei,* and *Lacticaseibacillus casei*. Additionally, *Acetobacter pasteurianus* has also been identified by Wang et al. (2022).

Lidums & Karklina (2014) observed that during kvass fermentation, LAB and yeasts populations dominate over other microorganisms, with aerobic microorganisms decreasing rapidly. (Dlusskaya et al. (2008) suggested the use of *Lactococcus lactis* var. *diacetilactis* as a starter culture in combination with *S. cerevisiae* to improve aroma and organoleptic properties of kvass.

Microbial composition significantly impacts the quality of kvass, despite the availability of commercial starters differing from traditional origins. Few studies have explored microbial associations in kvass fermentation (Wang et al., 2022), emphasizing the need for comprehensive research on the natural microbial flora in the raw materials used in kvass production.

1.3 Rye cereal

Rye belongs to the grass family Gramineae and the genus Secale, with *Secale cereale* being the most widely cultivated species. A minor amount of *S. fragile* is also grown in southwestern Asia. The genus Secale includes five species: *S. silvestre, S. vavilovii*, *S. montanum*, *S. africanum*, and *S. cereale* (Miedaner, 1997).

Rye, unlike wheat, lacks evidence of cultivation in ancient Egyptian monuments or mention in ancient texts. Early northern European writings suggest it was initially cultivated in this region. Today, rye is grown globally, with major producers including Poland, Germany, western Russia, Belarus, and Ukraine (Dziki, 2022).

This crop is known for its minimal nutritional requirements, making it suitable for cultivation in regions where soil and climate conditions are unfavorable for other cereals (Smolik, 2013). Rye grain is primarily composed of carbohydrates (56-70%), fiber (15-21%), protein (8-13%), lipids (2-3%), and ash (2%) (Brownlee et al., 2017). The main proteins in rye grain are albumins and prolamins (34% and 19%, respectively), followed by globulins (11%), and glutenins (9%) (Silventoinen et al., 2021).

Due to its protein content, rye flour acquires unique technological properties that support its use in various food manufacturing processes, despite its inability to form a continuous gluten network like wheat flour (Drakos et al., 2017).

Compared to wheat, rye flour contains relatively less starch and protein, but is richer in fiber content (Sluková et al., 2021).

Moreover, wholemeal rye flour has been found to exhibit several beneficial effects on health, due to its high dietary fiber content (Michalska et al., 2007). It also contains many bioactive compounds such as phenolic acids (PA), lignans, benzoxazinoids, fructans, β-glucans, resistant starch, and arabinoxylans (AX) (Pihlava et al., 2018; Sárossy et al., 2013).

The chemical composition of rye flour justifies its functionality, thereby designating it as a functional food additive whose properties can be transmitted to the final products. Rye is used in a variety of food products such as sourdough bread, crispbread, flakes, biscuits, snacks, and animal feed. The addition of rye's milling products to food stuff increases their health-promoting characteristics (Dziki, 2022).

Another aspect to consider is the anti-nutritional impact of rye, as the bioavailability of minerals in cereals is often limited due to the presence of anti-nutritional elements such as phytate, trypsin inhibitors, and polyphenols. Phytic acid, found in most grains, is the most significant anti-nutrient (Ismagilov et al., 2020).

It's presence in food can reduce the absorption of several dietary minerals. However, this adverse effect can be mitigated by reducing the consumption of phytic acid-rich grain products (Nadeem et al., 2010).
Moreover, fermentation of germinated rye, which lowers the pH compared to native rye flour, increases levels of folates, free phenolic acids, lignans, total phenolic compounds, and alkylresorcinols. The bioactive potential of whole meal rye can thus be further increased through fermentation (Ram et al., 2020).

1.3.1 *Secale montanum*

Secale montanum, a member of the Poaceae family (tribe Triticeae), is believed to be the ancestor of *S. cereale* L. It is a perennial, outcrossing wild grass that grows naturally in semi-arid and arid grazing lands. This species is commonly distributed in Europe, Africa, and Asia. *S. montanum* is cultivated in various rangeland worldwide and is also often selected for restoration projects due to its high protein nutritional value and is superior adaptability compared to other grass species (Daly et al., 2023; Miedaner, 1997).
The loss of biodiversity has been a crucial issue in the last century, with an estimated 75% of the species used for food and agriculture being lost (Hammer et al., 1996). Consequently, the safeguarding and promotion of agrobiodiversity have become central themes in recent decades (Jackson et al., 2007). Throughout history, farmers have played a crucial role in maintaining cereal agrobiodiversity through various practices aimed at preserving and enhancing genetic diversity within their crops (Jones et al., 2008).
Landraces are cultivated plant populations distinguished by their historical origins and unique characteristics, lacking commercial crop improvement. They exhibit locally adaptability, genetic diversity, and association with traditional farming (Villa et al., 2005).
As reported by De Carvalho (2013), "the introduction of modern cultivars, combined with the mass exodus of the young workforce from rural areas, led to the consequent abandonment of traditional agricultural practices". Nevertheless, cereal landraces are still considered important in agricultural production, particularly in marginal lands and mountain areas where hybrids lose their competitive advantage (Giupponi et al., 2021). The adaptability of landraces to unfavorable conditions contributes to yield stability (Tesemma et al., 1998).

1.3.2 *Rye bread*

Rye cereal is one of the most commonly used grains for the preparation of bread, second only to wheat (Bushuk, 2001). Among rye-based products, rye bread is the most popular. Compared

to whole wheat bread, rye bread has a denser and less porous crumb structure with a gel-like fragility (Ikram et al., 2023).

Most rye bread is prepared using a fermentation starter. The incorporation of rye sourdough in bread production is associated with the distinct attributes of the final products and their cultural importance. Sourdough traditionally involves blending rye flour with water and allowing it to ferment. Bakeries usually maintain their own starter through a back-slopping process. The microorganisms, primarily heterofermentative lactic acid bacteria, originate from the flour and effectively metabolize maltose and sucrose, which are sugars naturally present in rye flour. During the fermentation period, the enzymatic activity of the microflora produces flavor compounds, mainly lactic acid and acetic acid. After fermentation, more flour, water, and other ingredients are added to the sourdough to make the dough. The dough is left to rise for a short period, shaped, left to rise again, and then baked (Decock & Cappelle, 2005; Ikram et al., 2023).

1.3.3 *Rye production in Poland*

Rye was introduced in Poland in the 5th century B.C. Since then, the widespread cultivation of rye in Poland has been enhanced by its low environmental requirements, high capacity to absorb macro- and micronutrients from nutrient-poor soils, tolerance to acidic pH levels, resilience to low temperatures, and frost resistance. These factors make the soil conditions in Poland highly favorable for rye cultivation (Arseniuk & Oleksiak, 2003).

Figure 2 illustrates the hectares of rye production within Europe in 2023. As shown, Poland stands out as the leading producer in terms of both cultivation area (hectares) and overall production volume among the countries represented.

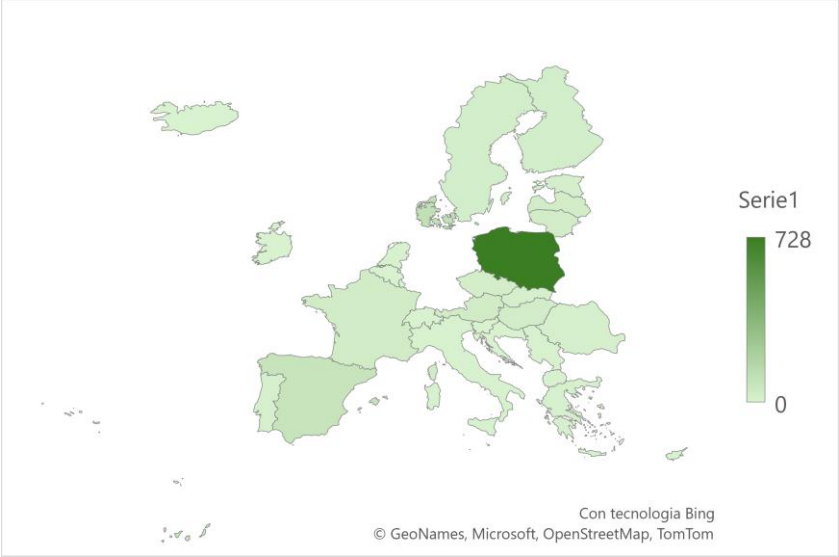

Figure 2: Area (cultivation/harvested/production) (1000 ha) of rye (2023), (Eurostat, 2023).

A more detailed trend of Poland's rye production is shown in Figure 3. Data for the years 2009-2011 and 2020-2022 are missing, possibly due to the lack of data provided by various producers. Overall, rye production in Poland has shown a decreasing trend since 2000.

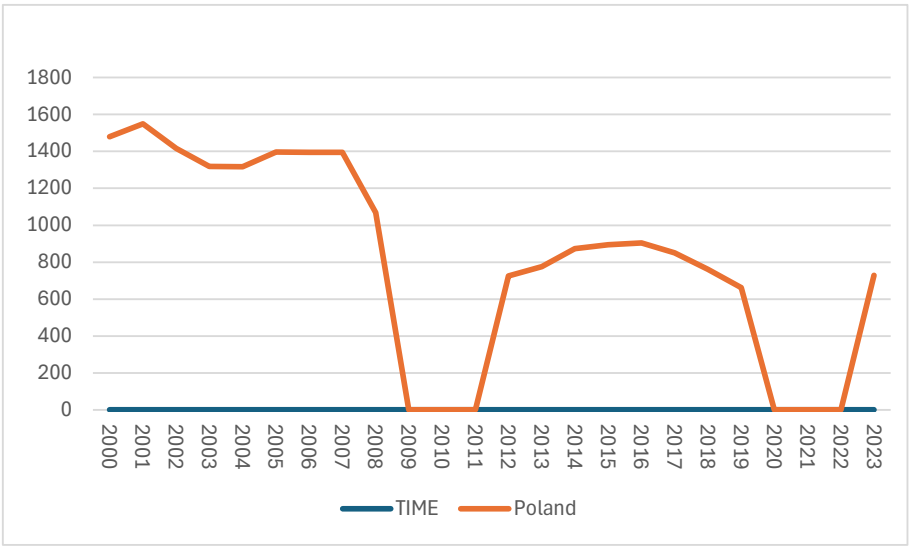

Figure 3: Area (cultivation/harvested/production) (1000 ha) of rye in Poland (2000-2023), (Eurostat, 2023).

Despite the decreasing trend, Poland remains the country with the highest rye production in 2023, as shown in Figure 3.

1.4 Food waste

Food waste is responsible for significant economic, environmental, and social impacts, making the implementation of reduction strategies critically important. A pivotal step in this effort was the establishment of Sustainable Development Goal (SDG) target 12.3, aiming to halve per capita global food waste by 2030 across retail, consumer levels, and throughout production and supply chains" (Lee et al., 2016). The European Commission has committed to achieving this target through initiatives outlined in the European Circular Economy Action Plan (European Commission, 2015). Food waste occurs at every stage of the supply chain, from farms and processing facilities to retails, restaurants, and households. The European Commission (2023) has listed the factors contributing to food waste:

- Insufficient shopping and meal planning;
- Shopping environment (e.g. promotions like "buy one, get one free" that may lead to impulse buying and over-purchase);
- Misunderstandings about the meaning of "best before" and "use by" date labels leading to edible foods being thrown away;
- Aesthetic considerations (bruised fruit and vegetables etc.);
- Standardized portion sizes in restaurants and canteens;
- Difficulty in anticipating the number of customers (a problem for catering services);
- Stock management issues for manufacturers and retailers;
- High quality standards (e.g. for produce sold at retail);
- Overproduction or lack of demand for certain products at certain times of the year;
- Production errors, products and/or labelling not meeting specifications;
- Product and packaging damage (farmers and food manufacturing);
- Inadequate storage/transport at all stages of the food chain including households (e.g. refrigerator temperatures);
- Busy lifestyle and conflicting priorities.

In the EU, more than 58 million tons of food waste are generated annually, averaging 131 kg per inhabitant (Eurostat, 2023). Households activities alone account for 54% of this waste, with the majority (approx. 70%) arising from households, food services, and retail (Eurostat,

2023). Effective waste management practices are imperative globally to address this issue comprehensively.

1.4.1 *Bread waste*

Due to its short shelf-life and overproduction, approximately 10% (900,000 tons) of bread is wasted throughout its supply chain, from manufacturer to consumer consumption, representing one of the highest contributors to food waste globally (Kumar et al., 2023).
The significant volume of bread waste reflects not only the loss of the product itself but also the depletion of various essential natural resources such as water, land, and energy involved in raw material production, transportation, and manufacturing (Narisetty et al., 2021).
Bread waste, like other forms of food waste, poses serious environmental challenges, including contributions to global warming, acidification, and eutrophication (Brancoli et al., 2020).
Therefore, reducing, recycling, or valorizing food waste has the potential to preserve global economy, conserve invaluable bioresources, and prevent millions of tons of greenhouse gases from entering the atmosphere (Kumar et al., 2023).

CHAPTER 2

Materials and methods

2.1 Rye flour

Rye flour (*Secale montanum*) was purchased from Ekohillar mill (Poland). Figures 4 displays the label of the flour package (a) and the flour sample (b). The proximate composition and nutritional values *S. montanum* flour are reported in Table 1.

(a) (b)

Figure 4: (a) label of *Secale montanum* flour from Ekohillar mill; (b) *S. montanum* flour.

Energy value	1381 kJ / 327 kcal
Fat of which saturated fatty acids	1.7% 0.3%
Carbohydrates of which sugars	56% 1.7%
Fiber	16%
Protein	14%
Salt	0%

Table 1: Nutritional values of *Secale montanum* flour.

2.2 Rye-bread making

A preliminary trial was performed on bread-making, to assess the feasibility of the bread production process, determine the optimal proportion of ingredients, and monitor the fermentation progress. Based on the preliminary results, a second trial was selected as definitely for the production and characterization of kvass.

For bread making, a 24-hour dough was prepared by initially mixing rye flour and water, which was then left to ferment at 25°C for 24 hours (Figure 5). Subsequently, this fermented dough was combined with additional rye flour, water, and salt (Figure 6).

Figure 5: Steps for the preparation of 24-hour dough.

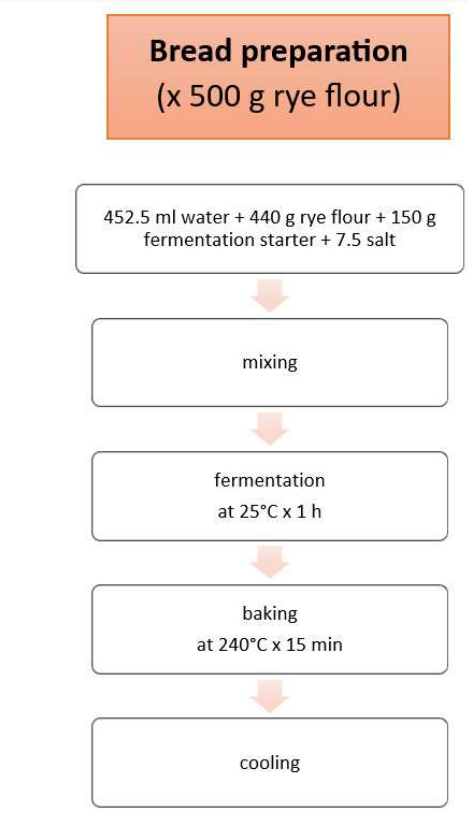

Figure 6: Flow chart of bread production.

Briefly, a fermentation starter dough was prepared by mixing water and *S. montanum* flour. Initially, this mixture was used as a leavening agent for bread. Later, using the same ingredients, it served as a starter for kvass production. Table 2 details the recipe, fermentation duration, and temperature used for the preparation of the starter.

Parameter	Amount	Unit of measure
Required fermentation stater dough yield	250	%
Rye flour	100	g
Water	150	g
Fermentation temperature:	30	˚C
Fermentation time:	24	h

Table 2: Ingredients for fermentation starter dough preparation.

All the ingredients were mixed under sterile conditions in a baker and covered with plastic film, as illustrate in Figure 7. The starter dough was labeled as “S”.

Figure 7: 24h-dough after fermentation used as a starter for bread-making.

All the ingredients were mixed and divided into small loaves, approximately 120 g each. The loaves were then fermented at 25 °C for 1 hour, followed by baking at 240 °C for 15 minutes. Figure 8 shows the bread loaves after baking.

Figure 8: Bread loaves after baking.

After baking, the bread was allowed to cool and then left to stale for 7 days at a refrigerated temperature (+4°C). Figure 9 compares the appearance of a bread half at two different times: first, just after baking (a), and second, after seven days (b).

(a) (b)

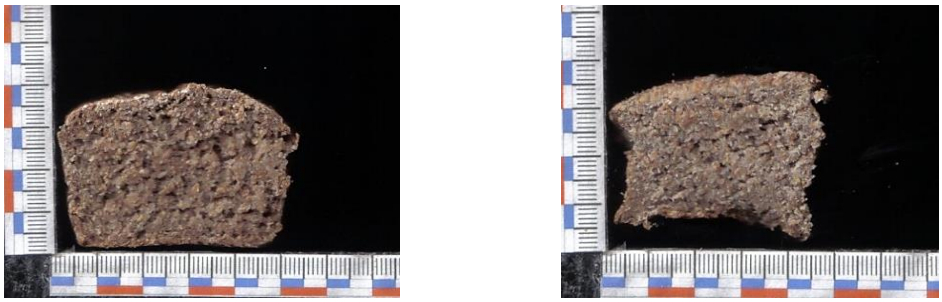

Figure 9: Scan of fresh bread (a) and bread after 7 days (b).

Before preparing the kvass, the bread was sliced, chopped, and then dried at 50°C for 72 hours.

2.3 Kvass

2.3.1 *Kvass preparation*

For kvass preparation, dried bread was added to hot water and brought to a boil, with occasional stirring. Afterward, the mixture was filtered to separate the liquid from the bread residues. Water was added to the mash to compensate for the loss during boiling, along with the 24h-dough, which had been prepared the day before using the same method as bread making. Once homogenized, the mash was divided into five 100 ml bottles of and ten 300 ml bottles. The smaller bottles were labeled SM1, SM2, SM3, SM4, and SM5 (Figure 10) and used to analyze the pH, color, and solid matter over the five days following bottling.

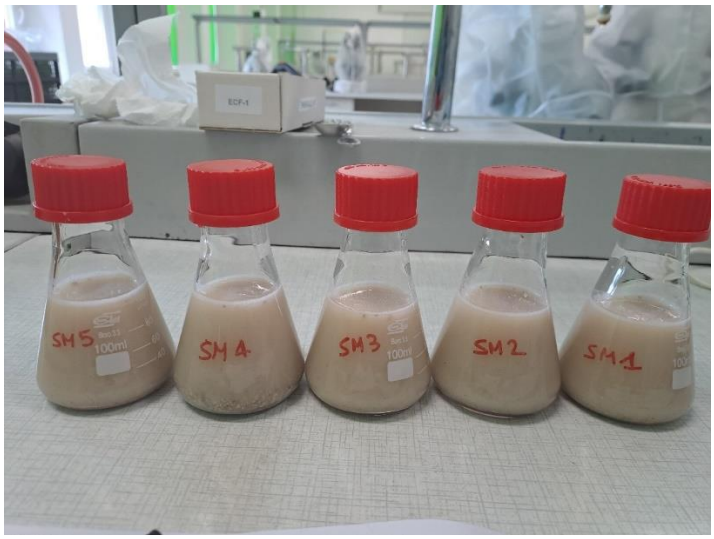

Figure 10: Laboratory-scale produced kvass.

The mash served as the substrate for fermentation, which occurred in bottles. As previously mentioned, two trials were carried out during this study. In the second trial, more water was used to obtain a higher amount of the final product and to simplify the filtration step. To prevent microbial contamination, all materials were pre-sterilized. Figure 11 shows the flow-chart of the laboratory-scale kvass production process.

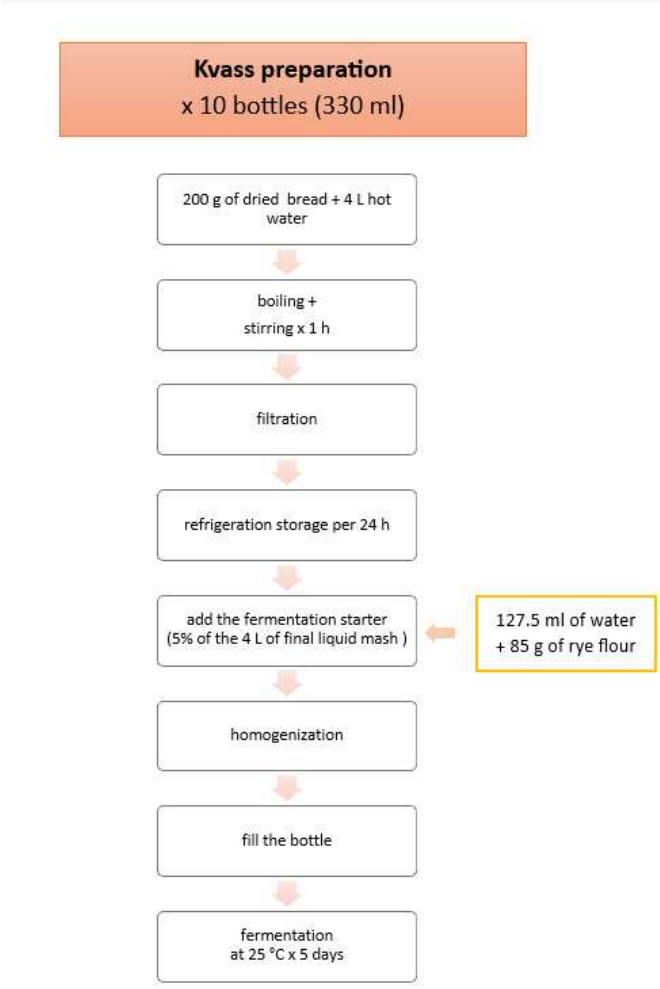

Figure 11: Flow chart of kvass-like preparation.

2.4 Color, solid matter, and pH measurements

Color analyses were performed using a colorimeter according to the CIELAB color space model (L*, lightness; a*, redness/greenness; b*, blueness/yellowness). The a* parameter represents the position between red (+a) and green (-a) on the color axis; positive a* values indicate reddish colors, whereas negative values indicate greenish colors. The b* parameter represents the position between yellow (+b) and blue (-b) on the color axis; positive values indicate yellowish colors, while negative values indicate bluish colors. The L* parameter represents lightness and ranges from 0 to 100, where 0 represents black, while 100 represents white. The midpoint value of 50 represents neutral grey (MacDougall, 2002).

For color analysis, an aliquot of kvass from each day of the study (from day 1 to day 5) was placed into a petri dish.

The solid matter was measured trough ATS60 moisture analyzer (AXIS Sp. z o.o., Gdańsk, Poland) by pouring an aliquot of kvass into an aluminum dish and let it to dry at 160°C until the achievement of a constant mass measure.

The pH was determined with a pH-meter equipped with a HI2031 solid electrode (Hanna Instruments, Padua, Italy) on 7-day-old bread. For each sample, measurements were taken in duplicate and the results expressed as mean ± standard deviation.

2.5 Viable counts

Viable counts were determined in kvass samples taken at different sampling times (t_0 until t_5). In detail, serial ten-fold dilutions were prepared from kvass samples using sterile peptone water (bacteriological peptone, 1 g L^{-1}, Oxoid, Basingstoke, UK). Aliquots of each decimal dilutions were inoculated in duplicate on the opportune growth media for the enumeration of the following microorganisms: (i) presumptive mesophilic lactococci on M17 agar incubated at 30°C for 72 hours; (ii) presumptive lactobacilli on De Man Rogosa and Sharpe (MRS) incubated at 30°C for 48 hours; (iii) yeasts on WL medium incubated at 25°C for 48-72 hours. The results were expressed as the log of Colony-Forming Units (CFU) per gram of each sample and reported as mean ± standard deviation.

2.6 Isolation and characterization of Lactic Acid Bacteria

2.6.1 *Isolation and identification*

Colonies grown on agar plates used for viable counting of lactic acid bacteria were randomly selected, sub-cultured to purity on the same media used for enumeration, and subsequently stored at -80°C for long-term maintenance. A total of 20 isolates were selected, with 10 isolates originating from the MRS medium (labeled as S1-S10) and another 10 isolates from the M17 medium (designated as S11-S20).

Total genomic DNA was extracted from the collected isolates using the protocol described by Osimani et al. (2015). Briefly, a sterile loop was used to aliquot and suspend viable biomass from each pure sub-culture into an Eppendorf tube containing 300 µL of TE buffer. The resulting solutions were then subjected to heat treatment in a Thermoblock FALC 1352 (Treviglio, Bergamo, Italy) at 100 °C for 10 minutes to ensure cell lysis. Following this, the samples were centrifuged at 13.000 rpm for 5 minutes, allowing the separation of the aqueous phase from the biomass, resulting in the formation of a precipitate at the bottom of the tube, and the supernatant containing the DNA, which was subsequently transferred into a new Eppendorf tube.

To evaluate the effective extraction of microbial DNAs, a PCR with the universal prokaryotic primers 27f and 1492r was performed. Amplification via PCR was carried out using the following primers: 27f [5′-AGAGTTTGATCMTGGCTCAG] and 1492r [5′-GGTTACCTTGTTACGACTT3′-AAGTCGTAACAAGGTAACC]. The PCR was performed using a MyCycler Thermal Cycler (BioRad Laboratories) in a final volume of 25 µL for each reaction tube, according to the reaction's conditions depicted in Figure 12.

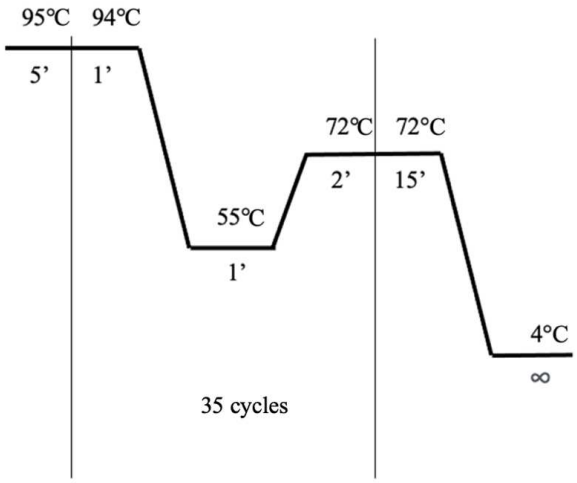

Figure 12: PCR amplification cycle.

The amplification was verified by electrophoresis by running the samples on a 1.5% (w/v) agarose gel in 0.5X Tris/Borate/EDTA (TBE) buffer containing 0.5 µg/mL GelRed® Nucleic Acid Gel Stain, 10,000X in water (Biotium, San Francisco, CA, USA). 5 µL of each amplicon were mixed with 2 µL of loading dye and located into the dedicated wells of the electrophoresis gel.

The electrophoretic run featured the HyperLadderTM 1 kb (Meridian Bioscience, Cincinnati, Ohio, USA) as a molecular weight standard and was carried out at 75 V for 3.5 hours and visualized under UV light (λ = 260 nm). The resulting images were captured using a Canon Powershot G9 camera (Canon Italia S.p.A., Milano, Italy). Correct amplification was verified by comparison with the reference ladder. The amplicons were then shipped to Genewiz (Takaley, UK) for their purification and sequencing. Finally, a BLAST search was exploited to compare the obtained sequences (FASTA) with 16S rRNA sequences of type strains from GenBank database.

2.6.2 *Acidification capacity*

Firstly, a sterile rye bread extract (SRBE) was prepared following the procedure described by Rathore et al. (2012) with minor adjustments. In detail, 800 g of rye flour were thoroughly mixed with 800 g of water, 10 g of salt, and 25 g of fresh brewer's yeast. The mixture was allowed to ferment at 30°C for 2 hours, then baked at 220°C for 30 minutes. After cooling, 400 g of chopped bread was suspended in 3.6 L (1:10 w v^{-1}). The mixture was subsequently sterilized by autoclaving at 121°C for 20 minutes; the resulting mash was centrifuged at 4,000

rpm for 5 minutes. 20 ml of the supernatant were then aliquoted into 50 ml Falcon tubes, and the sterilization step was repeated. The sterile supernatant was used as a liquid broth in subsequent experiments.

The acidification capacity of the isolates was assessed as previously detailed by Rampanti et al. (2023) with slight modifications. First, the isolates were retrieved from the frozen stored suspensions and sub-cultured twice on the same medium used for isolation. After growth, bacterial suspensions were centrifuged (4,000 g for 5 minutes) and the cell pellets were resuspended in physiological water (0.9% NaCl) to achieve a cell density of approximately 10^6 cells/ml. The acidification capacity of the lactic acid bacteria was then tested by inoculating 20 ml of SRBE with 1% (v v^{-1}) of the of the cell suspension. The samples were incubated at 30°C, and pH measurements were taken in sterile conditions at 4, 8, and 24 hours post-inoculation.

2.6.3 *Enzymatic activities*

Isolates with the best acidification performance were tested for key enzymatic activities, using the semi-quantitative, color-based micromethod API® ZYM (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Each API® ZYM (bioMérieux) strip is composed of 20 cupules containing synthetic substrates that are inoculated with the microorganism to be tested in a water suspension, this latter used to rehydrate the enzymatic substrates. The 20 cupules are designed to evaluate the activity of the following enzymes: 1 – control; 2 - alkaline phosphatase; 3 - esterase (C 4); 4 - esterase lipase (C 8); 5 - lipase (C 14); 6 - leucine arylamidase; 7 - valine arylamidase; 8 - cystine arylamidase; 9 - trypsin; 10 - alpha-chymotrypsin; 11 - acid phosphatase; 12 - naphthol-AS-BI-phosphohydrolase; 13 - alpha-galactosidase; 14 - beta-galactosidase; 15 - beta-glucuronidase; 16 - alpha-glucosidase; 17 - beta-glucosidase; 18 - N-acetyl-ß-glucosaminidase; 19 - alpha-mannosidase; 20 - alpha-fucosidase. The metabolic end-products produced during the incubation period were detected through coloured reactions revealed by the addition of reagents. Briefly, a suspension in API Suspension Medium (bioMérieux) (2 mL) of each isolate grown on MRS agar (Merck) was prepared according to a turbidity of 5–6 McFarland (bioMérieux). Sixty-five µL of the obtained suspension were used for the inoculation of each cupule of the API® ZYM (bioMérieux) strips with incubation for 4 h at 37°C. After incubation, 1 drop of ZYM A reagent (bioMérieux) and 1 drop of ZYM B reagent (bioMérieux) were added to each cupule until colour development (at least 5 min). For each cupule, a value ranging from 0 to 5 was assigned, corresponding to the colour developed: 0 corresponding to a negative reaction, 5 to a reaction

of maximum intensity and 1, 2, 3, or 4 were intermediate reactions depending on the level of intensity (3, 4, or 5 being considered as positive reactions).

2.6.4 *Antimicrobial activity*

The antimicrobial activity was evaluated using the agar well diffusion assay, following the method previously outlined by Cardinali et al., (2024). Prior to testing, the isolates were retrieved from frozen-stored suspensions and sub-cultured twice at 30 °C for 48 hours, followed by a third sub-culturing step for 24 hours. After collecting a 500 µL aliquot of the bacterial culture, the remaining broth cultures underwent centrifugation at 1,610 × g for 10 minutes. The supernatant was then neutralized to pH 7 using 0.1 N NaOH (AppliChem, Darmstadt, Germany), followed by filtration through a sterile PES membrane filter with a pore size of 0.22 µm (Laboindustria S.p.A., Padova, Italy).

The Brain Heart Infusion (BHI) soft agar growth medium (VWR) was inoculated with a concentration of 2% (v/v) of the target microorganism *Listeria innocua* and poured into 90 mm diameter Petri dishes. After solidification, wells of approximately 50 µL were created using a sterile 200 µL tip cone. For each isolate, four wells were prepared, containing: (i) 50 µL of the sub-cultured suspension; (ii) 50 µL of the neutralized suspension; (iii) 50 µL of the filtered neutralized suspension; and (iv) 50 µL of sterilized water as a negative control.

The Petri dishes were then incubated at 37 °C for 24 hours and examined for the presence of inhibition halos.

2.6.5 *In-vitro EPS production*

To detect in vitro EPS-producing isolates, the method previously described by Rampanti et al., (2024) was used, with few adjustments. Initially, the isolates were retrieved from cryo-protective suspensions and sub-cultured twice at 30 °C for 48 hours. Following this, 5 µL of each bacterial culture was added in duplicate on the following solid media: (i) MRS agar supplemented with 80 g L^{-1} sucrose to promote the synthesis of homopolysaccharides (HoPSs); (ii) MRS agar supplemented with yeast extract (VWR Chemicals) (10 g L^{-1}), meat extract (10 g L^{-1}), lactose (Carlo Erba, Cornaredo, Italy) (20 g L^{-1}), and galactose (VWR Chemicals) (20 g L^{-1}) to promote the synthesis of heteropolysaccharides (HePSs). After a 48-hour incubation at 30 °C, the colonies were classified as positive whenever presenting a ropy consistency (able to produce visible filaments with a sterile toothpick) or a mucoid appearance (displaying a glossy, slimy texture).

2.6.6 *Detection of hdcA gene of Gram-positive bacteria on microbial isolates*

The presence of the histidine decarboxylase (*hdcA*) gene in the isolates was carried out in a CFX Connect Real-Time System machine (BioRad, Hercules, CA, USA). The cycling conditions and primers used were as described by Belleggia et al. (2021), targeting a 174 bp fragment of the *hdcA* gene (Fernández et al., 2006). *Lactobacillus parabuchneri* DSM 5987 was used as a positive control to create the standard curve. Each isolate was analyzed in duplicate along with a blank control. The results were reported as either the presence (+) or absence (−) of the *hdcA* gene in the bacterial DNA samples.

2.7 Statistical analyses

Statistically significant differences in color, pH, solid matter, and microbial counts at different sampling times were assessed using the Tukey-Kramer's Honest Significant Difference (HSD) post-hoc test with a significance level of 0.05 following one-way analysis of variance (ANOVA). Tests were conducted using JMP v11.0.0 software (SAS Institute Inc., Cary, NC).

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Kvass characterization

3.1.1 *Color*

The results of the colorimetric analysis of the samples at five different times are reported in Table 4. The results revealed significant changes in the lightness (L*), red/green value (a*), and yellow/blue value (b*) of the samples over time. Overall, a wide variability was observed in all parameters at different times.

In more detail, the lightness (L*) showed values ranging from 49.43 ± 0.29 (t3) to 83.83 ± 2.08 (t5), indicating a higher lightness after five days of fermentation compared to the other times.

Concerning a* parameter, the values ranged between 0.57 ± 0.12 (t2) and 3.27 ± 0.31(t4). At t1, the value was significantly higher compared to t5 (1.55 ± 0.07 *vs* 0.60 ± 0.17). However, the values did not decrease progressively during fermentation, and the a* value reached its highest point (3.27 ± 0.31) at t4.

As for b* parameter, similar values were observed during the first three days of fermentation, ranging from 9.07 ± 0.76 to 11.33 ± 1.21. At t4, b* reached the highest value (14.07 ± 0.75), followed by a significant decrease at t5.

Time [days]	L*	a*	b*
1	50.20 ± 1.13 ^{B}	1.55 ± 0.07 ^{B}	9.95 ± 0.64 ^{B}
2	54.10 ± 0.36 ^{B}	0.57 ± 0.12 ^{C}	9.07 ± 0.76 ^{B}
3	49.43 ± 0.29 ^{B}	1.57 ± 0.38 ^{B}	11.33 ± 1.21 ^{A,B}
4	55.77 ± 0.50 ^{B}	3.27 ± 0.31 ^{A,B}	14.07 ± 0.75 ^{A}
5	83.83 ± 2.08 ^{A}	0.60 ± 0.17 ^{C}	5.90 ± 0.30 ^{C}

Table 3: Results of colorimetric analyses of kvass-like beverage expressed in terms of L* (brightness), a* (red/green value) and b* (yellow/blue value) at five different times (days). Values are expressed as mean ± standard deviation of three measurements. In each column, different superscript letters indicate significant differences between samples at different times (P<0.05).

3.1.2 *Determination of pH and solid matter*

The results of pH and solid matter during 5-day fermentation of kvass are reported in Table 4. Concerning pH, at day 0, the initial pH was 3.39, indicating an already acidic environment. After 24 hours, there was a significant increase in pH (3.89 ± 0.06), suggesting a substantial shift in the chemical environment. However, by day 2, the pH decreased again to 3.5, approaching the initial value. Between days 2 and 5, no significant differences were observed, with pH values ranging from 3.39 to 3.50.

In this study, a spontaneous fermentation was conducted without the use of starter cultures.

In the study by Pisponen and Andreson (2024) on kvass, the initial pH value was 5.58 ± 0.13, significantly higher compared to the values herein observed. Throughout the fermentation period, a significant decline in pH values was observed, attributed to the accumulation of organic acids produced by the LAB cultures (Pisponen and Andreson, 2024). By contrast, the stability of the pH values observed in this study suggests that fermentation had already occurred before bottling.

Time [days]	**pH**	**Solid matter [g 100 g^{-1}]**
0	3.39 ± 0.07 ^{B}	4.70 ± 0.02 ^{B}
1	3.89 ± 0.06 ^{A}	4.20 ± 0.03 ^{C}
2	3.50 ± 0.07 ^{B}	4.80 ± 0.01 ^{B}
3	3.49 ± 0.02 ^{B}	5.10 ± 0.07 ^{A}
4	3.40 ± 0.02 ^{B}	4.40 ± 0.02 ^{B,C}
5	3.39 ± 0.02 ^{B}	4.70 ± 0.20 ^{B}

Table 4: Results of pH and solid matter at different times (days). Values are expressed as mean ± standard deviation of x measurements. In each column, different superscript letters indicate significant differences between samples at different times (P<0.05).

As for solid matter, the values ranged between 4.2 ± 0.03 (t1) and 5.1± 0.07 (t3) g 100 g^{-1}. At time 0, the solid matter accounted for 4.7 ± 0.02 g 100 g^{-1}. After 24 hours, a statistically significant reduction in solid matter content was observed compared to t0. However, the value increased again at t2, reaching the highest value at t3. From day 4 to day 5, the solid matter returned to the initial value. Higher solid matter values are reported in the literature for kvass samples; Lidums et al. (2015) found values of 8.6%, 7.9%, 12.1% in different kvass beverages. The solid matter content is clearly related to the amount of water present in the samples. These

results suggest that the kvass samples produced in this study contained more water compared to the samples analyzed in the literature.

3.1.3 *Viable counting*

The results of viable counts of kvass samples during fermentation are reported in Table 5. Overall, high loads of presumptive lactococci, presumptive lactobacilli, and yeasts were found in kvass-like beverage starting from time 0. All microbial groups under investigation showed relatively stable values over the five-day fermentation. In detail, the load of presumptive lactococci ranged between 8.13 ± 0.03 and 8.29 ± 0.04; similarly, the load of presumptive lactobacilli ranged between 8.22 ± 0.10 and 9.07 ± 0.07. A slight decrease of yeasts counts was observed at t4 and 5 (6.79 ± 0.07 and 7.83 ± 0.02, respectively

Day	Presumptive lactococci	Presumptive lactobacilli	Yeasts
0	8.29 ± 0.02 ^{A}	8.29 ± 0.01 ^{A}	8.52 ± 0.01 ^{A}
1	8.30 ± 0.09 ^{A}	8.31 ± 0.05 ^{A}	8.56 ± 0.25 ^{A}
2	8.15 ± 0.01 ^{A}	8.22 ± 0.10 ^{A}	8.59 ± 0.47 ^{A}
3	8.29 ± 0.01 ^{A}	9.07 ± 0.00 ^{A}	8.39 ± 0.20 ^{A}
4	8.29 ± 0.04 ^{A}	8.96 ± 0.00^{A}	6.79 ± 0.07 ^{C}
5	8.13 ± 0.03 ^{A}	8.73 ± 0.03 ^{A}	7.83± 0.02 ^{B}

Table 5: Results of viable plate counts (expressed in log cfu g^{-1}) in kvass-like beverage. Values are expressed as mean ± standard deviation. In each column, different superscript letters indicate significant differences (P<0.05).

The initial load of all microbial groups under investigation was higher compared to the values reported in the study of Lidums & Karklina (2014). Similarly to pH values, these results suggest that fermentation likely occurred before bottling.

Lactic acid bacteria and yeasts, the primary microorganisms involved in kvass fermentation, play crucial roles in developing its characteristic taste and aroma. The lactic acid produced during fermentation leads to a low pH of kvass, acting as a natural preservative that helps inhibit the growth of undesirable microorganisms thereby increasing the shelf life and product (Lidums & Karklina, 2014; Phiri et al., 2019). The literature suggests that yeasts and lactic acid bacteria (LAB) form a symbiotic relationship (Ramos et al., 2011). LAB create an acidic environment optimal for yeast activity, while yeasts produce essential amino acids and vitamins for microbial growth. However, both LAB and yeasts compete for nutrients. As

fermentation progresses, the reduction in dry matter and increased acidity favors the growth of LAB.

3.2 Identification and characterization of Lactic Acid Bacteria

Table 6 shows the results of the isolation and characterization of lactic acid bacteria isolated from kvass samples. A total of seventeen isolates were identified.

The isolates were identified as follows: *Pediococcus pentosaceus* (10), *Pediococcus acidilactici* (1), *Liquorilactobacillus nagelii* (2), *Latilactobacillus fuchuensis* (1), *Latilactobacillus curvatus* (2), and *Fructilactobacillus lindneri* (1).

In a previous study by Dlusskaya et al. (2008), *L. casei* and *L. mesenteroides* were identified in kvass samples. However, the differences in bacterial identification can be attributed to the different raw materials used as ingredients in kvass formulation, their origins, and the differences in the production process.

P. pentosaceus is a lactic acid bacteria belonging to the Lactobacillaceae family. It produces lactic acid through the fermentation of sugars, and it's known for its acid tolerance. Notably, *P. pentosaceus* has been used in the fermentation of cereal-based products, as reported by Xing et al. (2019). This suggests its potential as a suitable starter culture for kvass production, leveraging its antimicrobial and fermentative properties to improve the quality and safety of the final product.

-, negative; +, positive colonies; sucrose-dependent, HoPSs; sucrose-indepentent, HePSs.

Isolate code	Closest Relative	Antimicrobial Activity	*hdcA gene*	EPS production	
				Sucrose-dependent	Sucrose-independent
S1	*Liquorilactobacillus nagelii*	-	-	-	-
S2	*Pediococcus pentosaceus*	-	-	+	-
S3	*Fructilactobacillus lindneri*	-	+	+	-
S4	*Latilactobacillus curvatus*	-	-	-	-
S5	*Pediococcus pentosaceus*	-	+	-	-
S6	*Liquorilactobacillus capillatus*	-	-	-	-
S8	*Latilactobacillus fuchuensis*	-	-	-	-
S9	*Latilactobacillus curvatus*	-	-	-	-
S10	*Pediococcus pentosaceus*	-	-	+	+
S11	*Pediococcus pentosaceus*	-	+	+	+
S12	*Pediococcus acidilactici*	-	+	-	+
S13	*Pediococcus pentosaceus*	-	-	-	-
S14	*Pediococcus pentosaceus*	-	-	+	+
S15	*Pediococcus pentosaceus*	-	-	+	+
S18	*Pediococcus pentosaceus*	-	-	+	-
S19	*Pediococcus pentosaceus*	-	-	+	-
S20	*Pediococcus pentosaceus*	-	-	+	-

Table 6: Identification and characterization of lactic acid bacteria isolated from kvass.

3.2.1 *Assessment of antimicrobial activity*

Regarding the antimicrobial activity, none of the isolates showed antimicrobial activity against *L. innocua* (Table 6*).* However, as mentioned by Perez et al. (2022), the ability of lactic acid bacteria to produce bacteriocins varies significantly between strains and is influenced by the expression of multiple genes.

In the study by Fugaban et al. (2022), *Pediococcus pentosaceus* and *Pediococcus acidilactici* were found to produce bacteriocins with high specificity against *Listeria* and vancomycin-resistant *Enterococcus* species. Additionally, these strains were evaluated for their production of effective antifungal metabolites with potential for inhibiting mycotoxin-producing molds. This demonstrated antimicrobial activity suggests the potential of these strains in other food applications. Future research should continue to explore the conditions under which these strains might express antimicrobial activity and their potential applications in food safety and preservation.

3.2.2 *In Vitro EPS Production*

Exopolysaccharides (EPS) are extracellular polymers with important techno-functional and biological properties (Tieking et al., 2005). They are biosynthesized by a wide range of

bacteria, including lactic acid bacteria. EPS can be classified into heteropolysaccharides (HePSs) and homopolysaccharides (HoPS). HoPSs consist of a single monosaccharide (mostly fructose or glucose) and are usually produced in large amounts from sucrose. In contrast, HePSs are composed of two to eight monosaccharides (mostly glucose, galactose, rhamnose, and fructose) and are produced in lower amounts (Korakli & Vogel, 2006).

Incorporating EPS-producing LAB into starter cultures rather than using polysaccharide additives as texture enhancers, stabilizers, emulsifiers, gelling agents, or water-binding agents, could offer advantages in fermented foods (Smitinont et al., 1999).

In this study, EPS production was detected in several isolates from kvass samples (Table 6). Eight isolates of *P. pentosaceus* (S2, S10, S11, S14, S15, S18, S19, S20) and one isolate of *Fructilactobacillus lindneri* (S3) exhibited in vitro EPS production on MRS agar supplemented with sucrose (HoPSs). Additionally, four *P. pentosaceus* (S10, S11, S14, S15) and one *P. acidilactici* (S12) isolates were able to produce EPS on MRS agar enriched with yeast extract, meat extract, lactose and galactose (HePSs).

These findings are in accordance with the study conducted by Smitinont et al. (1999), which screened LAB isolated from various fermented foods for their EPS production capabilities. Their research indicated that several LAB strains, including *P. pentosaceus*, showed particularly high EPS yields, highlighting the potential of these strains to enhance the texture and stability of fermented products.

3.2.3 *Presence of hdcA gene,*

The presence of the *hdcA* gene was revealed by qPCR in the isolates *Fructilactobacillus lindneri* (S3), and *Pediococcus pentosaceus* (S5, S11, and S12) (Table 6).

The *hdcA* gene encodes the enzyme histidine decarboxylase, which catalyzes the decarboxylation of the amino acid histidine into histamine. Histamine is a biogenic amine that can significantly impact human health, potentially causing allergic reactions and symptoms of food poisoning if present in high concentrations in fermented foods.

The *hdcA* gene is often studied in relation to lactic acid bacteria (LAB) used in food fermentation because certain LAB strains may possess this gene and produce histamine during fermentation. The presence of histamine-producing bacterial strains in fermented products can pose a food safety risk, making it important to monitor and control histamine levels in these foods (Diaz et al., 2016). Therefore, this analysis becomes necessary for the safety assessment of LAB for their potential application as starter cultures in food fermentation processes.

3.2.4 *Acidification performance*

The results of the acidification performance of lactic acid bacteria isolated from kvass-like beverages are reported in Figure 13.

The initial pH of the sterile rye bread extract (SRBE) was 6.91. The ΔpH after 4 hours of fermentation ranged from 0.03 to 0.55, with the lowest pH value achieved by *Pediococcus pentosaceus* S20. The most effective acidification after 4 hours was observed in *P. pentosaceus* isolates S14, S18, and S20.

After 8 hours, the ΔpH varied from 0.6 to 1.71, with *P. pentosaceus* isolates S2, S11, and S15 demonstrating a higher acidification rate, reaching a pH range of 4.9 to 5.6.

After 24 hours, the acidification rate ranged from 0.34 to 2.03, with *P. pentosaceus* isolates S2, S13, and *Latilactobacillus curvatus* isolate S9 exhibiting the highest acidification rate, resulting in the lowest pH value of 4.12. By the end of the fermentation process, all isolates showed acidifying activity. The highest total ΔpH was 2.79, recorded for *P. pentosaceus* isolate S11.

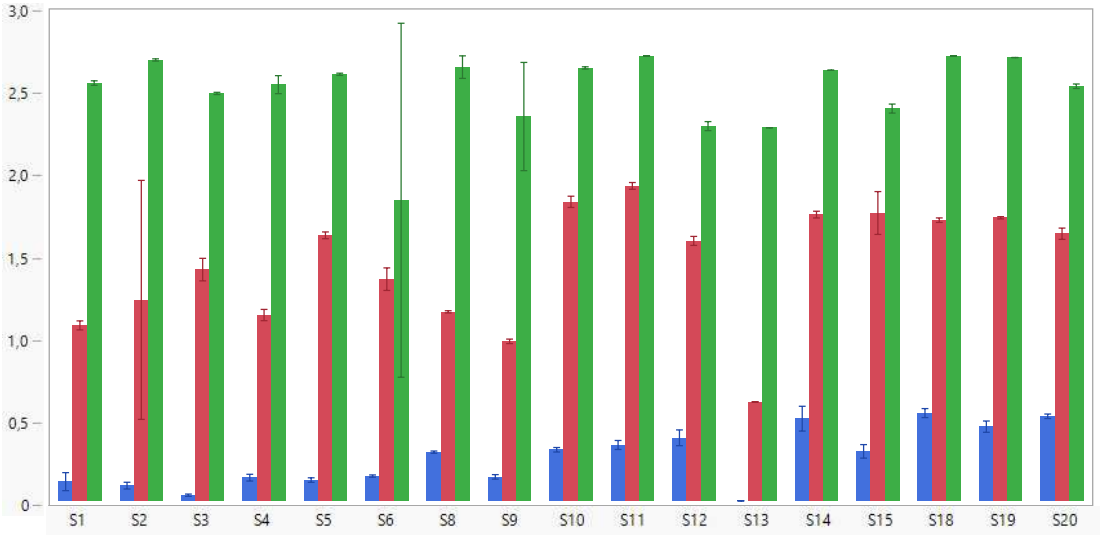

Figure 13: Acidification performance of the lactic acid bacteria isolated from kvass-like beverages after 4 h (blue bar), 8 h (red bar), and 24 h (green bar) of fermentation.

Recent studies have investigated the selection and evaluation of lactic acid bacteria for improving sourdough bread quality. For instance, Lancetti et al. (2021) highlighted the importance of identifying effective bacterial strains. This study focused on isolating and characterizing LAB from local grains, aiming to select strains with optimal fermentation properties by assessing their acidification rates and production of organic acids. The selected

LAB strains showed promising results in terms of enhancing dough properties, flavor development, and shelf life of the bread.

Similarly, Plessas et al. (2020) studied *P. pentosaceus* for its acidification ability in sourdough bread making, demonstrating its suitability for this application. This efficiency in acid production translated into superior bread quality, characterized by enhanced acidity, improved organic acid profiles, and increased resistance to spoilage compared to sourdough bread made with natural microbiota. These results suggest promising avenues for commercialization, where *P. pentosaceus* could be marketed as a dry, lightweight, and shelf-stable immobilized starter. Such advancements hold significant potential for industrial applications seeking reliable and efficient sourdough fermentation solutions.

3.2.5 *Enzymatic activities*

The assessment of key enzymatic activities was performed on lactic acid bacteria isolates that showed the best acidification performance and tested negative for the presence of the *hdcA* gene The selected isolates were S10, S14, S18, and S19.

The results of API-ZYM tests are reported in Figure 14. None of the isolates exhibited a positive reaction for alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), trypsin, alpha-chymotrypsin, alpha-galactosidase, beta-glucuronidase, alpha-glucosidase, N-acetyl-ß-glucosaminidase, alpha-mannosidase, and alpha-fucosidase. Conversely, all isolates showed activity for leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and beta-glucosidase. As for leucine arylamidase and valine arylamidase, all isolates exhibited strong activity. Weak cystine arylamidase activity was observed for *P. pentosaceus* S10 and S14. Additionally, weak activity for beta-glucosidase was observed for all the isolates. Overall, the highest enzymatic activities observed in all isolates were for leucine arylamidase and valine arylamidase.

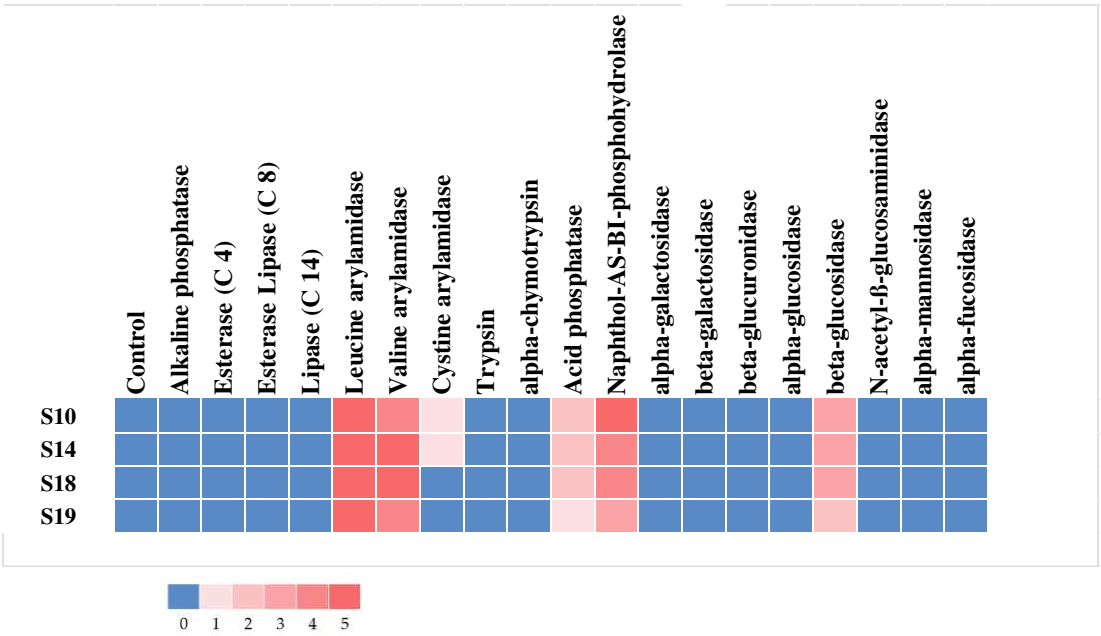

Figure 14: Heat map representing the results of semi-quantitative assessment of enzymatic activity of lactic acid bacteria isolated from kvass-like samples.

In the scientific literature, there are no studies related to the enzymatic activities of *P. pentosaceus* in substrates comparable to kvass. However, it is known that *P. pentosaceus* is isolated from a wide variety of sources. In particular, studies have shown that in dairy products *P. pentosaceus* exhibits high peptidase activities (leucine arylamidase and valine arylamidase) (Tzanetakis & Litopoulou-Tzanetaki, 1989).

CONCLUSIONS

The popularity of traditionally non-alcoholic beverages has varied across different historical periods and among different cultures. Originating in Eastern Europe, kvass has evolved over centuries as a fermented drink primarily made from rye staled bread. The production of kvass can be distinctly categorized between traditional methods, which involve spontaneous fermentation, and more industrialized approaches aimed at large-scale production. In any case, kvass represents an example of how fermentation can help reduce food waste by transforming a waste product, such as stale bread, into a new and valuable food product.

However, scientific research on kvass optimization and the microorganisms involved in its production remains limited to date. This study focused on the laboratory-scale production of kvass from homemade wholemeal rye bread. Kvass-like beverages were produced using a traditional method through spontaneous fermentation. The samples were assessed by physico-chemical and microbiological analyses. Moreover, a total of seventeen lactic acid bacteria were isolated from the kvass-like beverages and characterized in order to select potential starter cultures for kvass or other cereal-based non-alcoholic beverages. The experimental results diverged from those reported in the limited existing literature, highlighting areas for further exploration. Notably, the lactic acid bacteria identified in this study in kvass samples differed significantly from those found in commercially available products. The main isolates included *Pediococcus pentosaceus* and minor lactobacilli. Isolates S10, S14, S18, and S19 (*P. pentosaceus*) showed the best properties to be used as potential starter cultures. The selection was based on their acidification performance and absence of the *hdcA* gene encoding for histidine-decarboxylase.

Overall, this study contributes to the valorization of rye in Polish culinary heritage, promotes sustainable practices by repurposing staled bread, and enhances scientific understanding of traditional fermentation processes and microbial ecosystems in fermented beverages. Future studies could explore optimizing fermentation conditions to align microbiological profiles more closely with commercial standards while maintaining the authenticity of traditional kvass production methods.

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