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Decolorized and Undecolorized Ethanol Extracts of Ginggiyang (*Leea aequata* L.) Leaves as Effective Preservatives for Edible Products

Running head: Extracts of *Leea aequata* L. as Preservatives

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SUMMARY

Research background. The global increase in population has led to a corresponding rise in the production of edible products and chemical preservatives. However, chemical preservatives are often associated with adverse health effects, underscoring the need to develop safer and more effective alternatives.

Experimental approach. This research assessed the effectiveness and sensory acceptability of decolorized and undecolorized ethanol extracts of *Leea aequata* L. leaves as a preservative. The assessment was carried out by applying extracts to bread, jam, and juice products, while hedonic tests were also conducted to determine sensory acceptability. The total plate count (TPC) and yeast

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mold (YM) count were selected to evaluate the effectiveness of preservation. A comparison was performed with a control without additional preservatives and products preserved using potassium sorbate.

Results and conclusions. Products treated with test extracts were as sensorily acceptable as those treated with potassium sorbate. The addition of the treatment was proven to have a preservation effect, where the TPC and YM count were lower than in the control. Therefore, the test extracts had the potential to be developed as natural preservatives that could replace chemical variants, such as potassium sorbate.

Novelty and scientific contribution. Decolorized and undecolorized ethanol extracts of *Leea aequata* L. leaves had only been shown as preservatives of edible products for the first time. The results of these tests were crucial in developing effective preservatives.

Keywords: hedonic test; *Leea aequata* L.; natural preservatives; total plate count; yeast and mold count

INTRODUCTION

The continuous growth of the global population has led to an increasing demand for food products. Consequently, innovations in food production must be accompanied by the development of new additives, including preservatives. In this context, the pursuit of safer, more effective, and sustainable preservative alternatives has become a pressing priority (*1–4*). The demand for the discovery of natural preservatives, specifically those derived from plants, is also increasing (*5,6*).

According to previous research, microbial contamination of food is the leading cause of the need for preservatives (*7*). This contamination can cause spoilage of products and various foodborne diseases. The World Health Organization (WHO) states that 600 million people fall ill from foodborne pathogens each year (*8*). Microbial contamination can lead to a range of foodborne illnesses, with symptoms varying from mild discomfort to life-threatening conditions (*9*). These conditions are generally caused by the contamination of food products by foodborne pathogens, such as bacteria, fungi, viruses, and parasites (*10*). Typical examples of pathogenic bacteria include *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *C. perfringens*, *Cronobacter sakazakii*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio* spp., and *Yersinia enterocolitica*. These microbes can be found in various forms, types, and properties. Some bacteria can form heat-resistant spores, including *C. botulinum* (*11*), *C. perfringens*, *B. subtilis*, and *B. cereus* (*12*). The bacteria can survive even when exposed to high temperatures. *S. aureus* and *C. botulinum* are also able to produce heat-resistant toxins (*11,13*). Meanwhile, pathogens, such as *L.*

monocytogenes and *S. enterica,* can grow in cold temperatures (*14*). The properties of these pathogenic bacteria are a problem in maintaining the quality of edible products, where some have the potential to evolve to be heat-resistant (*15*). This adaptation renders preservation technologies that rely solely on heat treatment ineffective, underscoring the need to develop new preservatives.

Globally, an estimated 300 fungal species pose health risks, the majority of which are foodborne pathogens (*16*). Several fungi excrete mycotoxins, which contaminate food and cause acute poisoning, cancer, liver disease, and other diseases (*17*). *Aspergillus*, *Candida*, *Alternaria*, and *Fusarium* are fungal genera that often become foodborne pathogens (*18,19*). Hepatitis A and Norovirus are included in the group of pathogenic viruses, while *Cyclospora cayetanensis*, *Toxoplasma gondii*, and *Trichinella spiralis* belong to the category of parasite pathogens (*10*).

Based on the results, preservatives commonly used in edible products in the community are chemicals added to food to slow down deterioration. Preservatives are additives that are allowed in food products, but their application must be regulated to ensure safety, and they should be produced in accordance with good manufacturing practices. Moreover, their addition must not exceed the required limit as stipulated in the regulations, as well as food-grade (*20–22*). The most commonly used preservatives are a group of weak acid compounds and their salts, such as sorbic acid (*23,24*) and benzoic acid (*25*). Although the compounds are included in the GRAS (generally recognized as safe) category (*24*). Continuous use in the long term can lead to resistance (*25*), which further increases the need for efforts to discover new preservatives.

Therefore, this research aimed to evaluate the effectiveness and acceptability of decolorized and undecolorized *Leea aequata* leaves ethanol extracts in preserving edible product samples. The potential of this extract as a preservative was initiated with a previously published antimicrobial activity test. The test proved that this extract has antimicrobial activity against several foodborne pathogens, both bacteria and fungi. Microbes that are sensitive to this extract include *Candida albicans*, *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *Aspergillus flavus* (*26*). Its effectiveness as a preservative was then tested on food samples by determining the total plate count (TPC) and yeast and mold count (YM), and the acceptance was tested using a hedonic test.

MATERIALS AND METHODS

The 96 % ethanol (Merck, Darmstadt, Germany), powder activated carbon (PAC) (Merck, Darmstadt, Germany), potassium sorbate (Jiangsu Mupro IFT Corp., Jiangsu Province, China), Avicel 102 (Dupon Pharma, Wilmington, USA), and aqua demineralization (Bratachem, Bandung, Indonesia) have been used in the research. The 3M™ Petrifilm™ Yeast and Mold Count Plate (Petrifilm YM) and 3M™ Petrifilm™ Aerobic Count Plate (Petrifilm AC) were obtained from Semesta Mulia Medika,

Indonesia (3M, Minnesota, USA). Buffered peptone water was obtained from Merck Partners, Indonesia (Merck, Darmstadt, Germany). The ingredients for making bread samples: flour (Indofood Sukses Makmur, Jakarta, Indonesia), sugar (Sugar Group Companies, Lampung, Indonesia), instant yeast (Lesaffre, Marcq-en-Baroeul, France), milk powder (Nestle, Pasuruan, Indonesia), cocoa powder (BT Cocoa, Tangerang, Indonesia), margarine (Upfield Manufacturing Indonesia, Bekasi, Indonesia), chicken eggs, and salt (Refina, Surabaya, Indonesia). Ingredients for making jam (pineapple, sugar, and salt), and juice (guava fruit, sugar) were obtained from local supermarkets in the city of Bandung, Indonesia.

Plant extracts preparation

The test extracts included decolorized (DE) and undecolorized (EE) ethanol extracts. The extracts were prepared by maceration, where 1 kg of simplicial powder of *Leea aequata* leaves was macerated with 3×5 L of 96 % ethanol. The solvent replacement was carried out every 24 h, and the extracts were collected and filtered, and then concentrated using a rotary evaporator (IKA RV-8, Saufen im Breisgau, Germany) until a viscous extract was obtained. The thick extract was dried with a small amount of microcrystalline cellulose (Avicel 102) in an oven (Memmert UF260, Schwabah, Germany) at 40 °C until constant mass. The constant mass extract was added to Avicel 102 until the final mass ratio of 2:3 was obtained. DE was made using the same maceration method as in EE. After the extracts were filtered, it was decolorized by adding 2 g of PAC to every 100 mL of extract. The extracts were stirred using a magnetic stirrer (IKA RCT Basic, Staufen im Breisgau, Germany) for 1 h, filtered, and concentrated using a rotary evaporator until a thick extract was obtained, which was dried using a 40 °C oven with a small amount of Avicel 102 and added back until the final ratio of the two substances was 1:1.

Application of extracts in bread samples

Every 1200 g of bread dough was made from high protein flour (500 g), granulated sugar (150 g), instant yeast (11 g), milk powder (52 g), cocoa powder (20 g), chicken egg (1 piece), water (260 g), margarine (100 g), salt (5 g), and preservatives. This was made in 6 formulas based on the difference in preservatives used. This included bread A without added preservatives, B with 1500 mg DE, C with 2500 mg DE, D with 1500 mg EE, E with 2500 mg EE, and F with 1000 mg potassium sorbate as comparative preservative. The bread roll was molded with a dough mass of about 14 g and then baked at 200 °C for 30 min. Bread products were packaged individually in plastic-oriented polypropylene (OPP) laminated size 6×12 cm (Indah Karunia Sukses, Jakarta, Indonesia) and stored at room temperature ((25±2) °C) for 12 days until the analysis was completed.

Application of extracts in pineapple jam samples

Jam was made by mixing grated pineapple (1 kg), sugar (100 g), salt (5 g), and preservatives. Jam A was without added preservatives, while B, C, and D contained 1000 mg DE, 1000 mg EE, and 1000 mg of potassium sorbate as a comparison preservative, respectively. Each mixture was then cooked over medium heat for 60 min. The product was packaged in plastic cups (Wingoh Albindo, Tangerang, Indonesia), 10 g per cup, and stored at room temperature ((25±2) °C) for 12 days until the analysis was completed.

Application of extracts in guava juice samples

Guava juice was made by blending 250 g of fresh guava, 50 g of sugar, 500 mL of water, and several preservatives: juice A was without added preservatives, while B, C, and D contained 750 mg DE, 750 mg EE, and 1000 mg of potassium sorbate as a comparison preservative, respectively. The product was packaged in plastic bottles (Indo Tirta Abadi, Tangerang, Indonesia), 50 mL per bottle, and stored in a refrigerator (Sharp SCH-405X-WH3, Karawang, Indonesia) at (4±2) °C for 12 days until the analysis was completed.

Organoleptic observation and hedonic test

Organoleptic observations (color, odor, texture, and presence/absence of fungal growth) were made on days 1, 3, 5, and 7, with taste observation on the first day.

The hedonic test was conducted with 40 untrained panelists, who were not informed about the ingredient composition of each food product. Each panelist evaluated six bread samples and four juice samples, and no incentives were offered for participation. The panelists were students of a university in Bandung aged 18–23 years, consisting of 20 male and 20 female panelists. The panelists had an average ability to distinguish and communicate organoleptic differences (color, taste, texture, and aroma) of the food samples tested. The participants were asked to give a general preference score using a 9-point hedonic scale, which included: 1=dislike extremely, 2=dislike very much, 3=dislike, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like, 8=like very much, and 9=like extremely (*27*). At each sampling session, panelists were asked first to clean their mouths (gargle) with mineral water, observe each sample's color, smell, taste, and texture, and give a score of overall acceptance.

Determination of yeast and mold count in food product samples

The determination was carried out using Petrifilm YM media. A total of 10 g of sample was put in a 100-milliliter glass beaker, then 0.1 % buffered sterile peptone water was added and the sample

was homogenized by sonication for 10 min. The sample solution was diluted until a dilution of 10^{-1}, 10^{-2}, and 10^{-3} were obtained, which was expected to produce yeast and mold values in the range of 25–250. The cover was opened on the Petrifilm YM plate, which was placed on a flat surface of the biosafety cabinet, and 1 mL of the sample of each dilution was dripped in the middle. The cover was then closed, and a sample droplet was placed on the top right side using a spreader and pressed. The sample was subsequently spread in a circular motion to matche the diameter of the spreader. Then the Petrifilm YM was incubated at 25 °C for 5 days with the cover at the top, with the number of plate stacks not more than 20. The plates were removed from the incubator on the fifth day, and the number of yeast and mold colonies was calculated. The yeast colonies were small, three-dimensional, of uniform size and pale pink to turquoise color. On the other hand, mold colonies were large, wide, flat, diverse in color, with a dark center (*28*). The number of yeasts and molds was determined at various times, at days 1, 3, 5 and 7.

Determination of total plate count in food product samples

The total plate count in food product samples was determined using Petrifilm AC plates. A mass of 10 g of sample was put in a 100-milliliter glass beaker, then 0.1 % buffered sterile peptone water was added and the sample was homogenized by sonication for 10 min. The sample solution was diluted to 10^{-1}, 10^{-2}, and 10^{-3}, expecting to yield a total plate count between 25 and 250. The cover was opened on the Petrifilm AC plate, which was placed on a flat surface of the biosafety cabinet, and the middle was inoculated with 1 mL of the sample at each dilution. The cover was then closed, and a sample droplet was placed on its right side using a spreader and pressed. The sample was subsequently spread in a circular motion to match the diameter of the spreader. The Petrifilm AC plates were incubated for 72 h at 30 °C (*29*). Afterwards, the plates were removed from the incubator and the number of colonies was calculated (*30*). The determination was carried out at different periods, namely days 1, 3, 5, and 7.

Statistical analysis

The results were expressed as the average standard deviation of at least three independent tests. This research data were statistically analyzed following an initial normality test. Data normality was tested using the Anderson-Darling method (*31*). Normally distributed data were analyzed using one-way ANOVA, while non-normally distributed data were analyzed using the Kruskal–Wallis test, followed by Tukey's or Dunn's Post Hoc test (*32*). Statistical analysis was carried out using Minitab v. 21 software (*33*), with a significance level of $p<0.05$.

RESULTS AND DISCUSSION

The administration of test extract and standard preservative (potassium sorbate) caused organoleptically observable differences in the resulting food products (Table 1). Food samples preserved with DE had almost the same texture, color and aroma as products that were not preserved or were preserved with potassium sorbate. Meanwhile, food samples preserved with EE had different texture, color and aroma. This effect is attributed to the high pigment content of EE, particularly chlorophyll, which is the primary pigment in leaves that plays a role in photosynthesis (*34*). Prolonged exposure to light causes degradation and bleaching of the chlorophyll color (*35*). The cooking process at high temperatures, such as bread baking and jam sample cooking, also accelerates chlorophyll damage, which then causes changes in the food texture and taste. The food products preserved by EE are reported to have an unpleasant color, texture and taste (*36*). In DE, most of the extract pigments have been removed, thereby the effects of chlorophyll were minimal.

Samples of bread and jam preserved with DE and EE maintained their quality until the 5th observation day. Physical changes were seen only on the 7th day, and mold growth began to appear on the 12th day in preserved bread samples and on the 7th day in preserved jam samples, in contrast to bread and jam without preservatives, where mold growth started from the 3rd day. This proved that the addition of EE and DE can maintain the quality of bread and jam for up to 5 days. However, bread and jam with EE preservatives had poor color and aroma. Organoleptic tests have proven that DE inhibited the growth of microbes in bread and jam samples, without affecting their color, taste and texture. In jam samples, it seemed that adding DE and EE was not as effective as potassium sorbate. The same change trend was observed in samples with and without DE and EE.

Preservative effects of EE and DE are a result of the antimicrobial effects of the plant extract. *Leea aequata* has indeed been proven to contain several antimicrobial compounds, such as scopoletin, vanillic acid, and kaempferol (*37*). Scopoletin has antibacterial (*38–40*), antifungal (*41*), antiparasitic, and even antiviral properties (*42*), while kaempferol has antibacterial (*43*) and antifungal (*44*) properties. Vanillic acid has been proven to have broad-spectrum antibacterial activity (*45*).

The hedonic test measured the level of consumer preference for a food product. This is one of the crucial tests in every food product development. The 9-hedonic point scale was rated the best for over 60 years, although other, more superficial scales were developed (*27*). Hedonic test was performed as part of sensory quality measurements (*46*), to determine the overall preferences of food products with EE or DE. Addition of EE or DE to the tested food products significantly affect the preference window compared to a product with no added preservatives. However, it was not significantly different from products preserved with potassium sorbate.

Differences in panelist preferences for bread and juice products preserved with test extracts were evaluated using the Kruskal–Wallis test, as the data were not normally distributed (*32*). This test showed a difference in the panelist preferences for the tested samples, and a post hoc analysis was performed to determine which test groups differed.

Addition of plant extracts as preservatives to bread and juice samples led to a decrease in the overall preferences (color, taste, texture, and aroma) compared to unpreserved products ($p<0.05$). However, there was no difference in the preferences between the food products with tested extracts and those with standard preservative (potassium sorbate). This means that the plant extracts could substitute the commonly used potassium sorbate (Fig. 1).

Total plate count and yeast and mold plate count are essential indicators in the food industry. This parameters can be used to predict the sanitary quality and shelf life stability of food products (*47*). They could also be used as an indicators of microbial contamination (bacteria or yeasts and molds, respectively) of food products, poor product processing, or even inadequate storage facilities (*48*). Total plate count is also known as standard plate count, aerobic mesophilic count, aerobic plate count, and aerobic colony count (*48*).

Fig. 2 shows that the total plate count of bread preserved with DE, EE or potassium sorbate was significantly lower than that of the unpreserved products. The total plate count values of samples without preservatives (bread A and juice A) increased significantly compared to the products with preservatives ($p<0.001$). This finding suggests that DE, EE and potassium sorbate effectively inhibited the growth of aerobic bacteria during storage. This pattern aligns with the research conducted by Atwaa *et al*. (*4*), who observed a reduction in microbial load in tomato paste and pasteurized milk products preserved with various plant extracts, *i.e.* fenugreek seed, sage leaf, thyme leaf, rosemary leaf and clove extracts.

The antimicrobial effects are thought to be linked to phenolic and flavonoid compounds in these extracts, which disrupt bacterial cell membranes and interfere with their metabolic enzymes (*3,43,44*). A previous study has shown that the leaves of *Leea aequata* contain various phenolic and flavonoid compounds (*49*), which supports this hypothesis.

Fig. 3 shows that the addition of DE, EE or potassium sorbate to bread, juice and jam samples caused a significant decrease in yeast count compared to unpreserved products ($p<0.001$). Similarly, Fig. 4 indicates that food products preserved with DE, EE or potassium sorbate exhibited significantly lower mold counts compared to unpreserved products. This clearly demonstrates the antifungal properties of DE, EE and potassium sorbate. The phenomenon of preventing yeast and mold growth in food products by the addition of sage, rosemary and clove extracts has been previously studied

(*4*). This research adds to the extensive list of plants that have the potential to be used as preservatives.

Both DE and EE inhibited the growth of bacteria and fungi in bread and juice samples with no significant differences observed ($p>0.05$). In jam samples, the YM count was 10^5 CFU/g sample on the 5th day of measurement, so the measurement on the 7th day was not carried out.

Using natural ingredients as preservatives has a longstanding history in food preservation practices. However, to date, no approvals have been granted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for any natural preservatives. Bread is frequently examined in studies assessing preservation effectiveness. This is primarily because it is essential to preserve bread, particularly when it is stored at room temperature (*50*). Natural ingredients that have been proven effective in preserving bread include raisin extract (*51*), mustard bran and flour (*52*), oregano (*Oreganum vulgare* L.) essential oil (*53*), and rosemary (*Rosmarinus officinalis* L.) essential oil (*54*). Although rosemary essential oil is effective in preserving food samples, its official use as a preservative has not yet been approved by the JECFA, unlike its extract, which has been approved for use as an antioxidant (*54*). The abundant potential of natural materials as preservatives, of which none has been approved for use internationally, is undoubtedly an interesting challenge in research.

CONCLUSIONS

Decolorized and undecolorized ethanol extracts of *Leea aequata* L. leaves were tested for their preservative effects in bread, jam and juice products. Both tested extracts inhibited the growth of microorganisms (bacteria and fungi), especially in bread and juice products. The decolorized extract provided more robust and sensorially acceptable antimicrobial effect, so it was concluded that it has a significant potential for further development as a viable natural preservative for edible products. The novelty of this work lies in the development, evaluation and diversification of the application of *Leea aequata* leaves, extending beyond their traditional use as preservatives for palm sap. This research is highly relevant in developing safe and effective plant-based preservatives and provides a promising foundation for further standardization, safety evaluation, and industrial application of *Leea aequata* extracts.

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ETHICAL APPROVAL

The study has received an ethical approval from the Health Research Ethics Commission at Bhakti Kencana University, with reference number 075/09.KEPK/UBK/VII/2023, dated July 20, 2023.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Purwaniati has participated in research design, data collection, analysis, and the preparation of article drafts. M.I. Iwo participated in the design of experiments, data interpretation, draft preparation, and draft revision. M. Insanu participated in the data interpretation, draft preparation, and critical review of the final version. R.E. Kartasasmita played a role in research design, data analysis, article drafting, and final review of the article.

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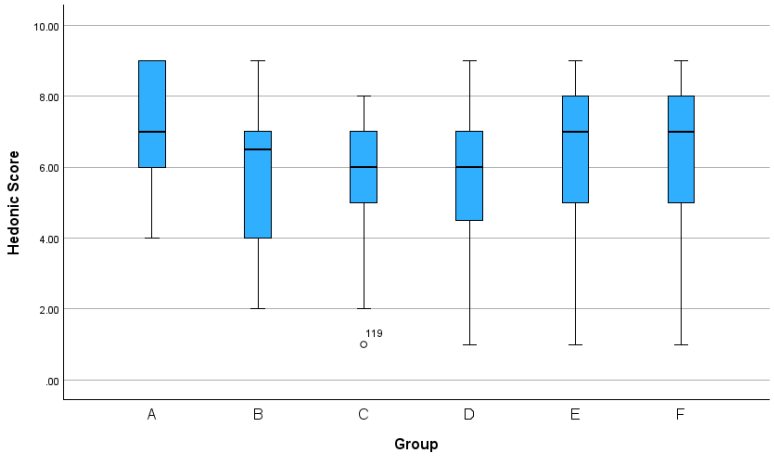
Table 1. Organoleptic observations of bread, jam, and juice samples without preservatives, and with decolorized (DE) and undecolorized (EE) ethanol extracts and potassium sorbate during storage

Sample	*t*(storage)/day				
	1	3	5	7	12
Bread A	Bread rises, characteristic aroma of bread, brown color	Same as day 1	Mold growth began to be seen	Molds are getting more and more	The mold covers almost the entire surface of the bread
Bread B	Bread rises, characteristic aroma of bread, brown color	Same as day 1	Same as day 1	Color fades	Less mold growth than bread A
Bread C	Bread rises, characteristic aroma of bread, brown color	Same as day 1	Same as day 1	Color fades	Less mold growth than bread B
Bread D	The bread is very fluffy in the oven, but it deflates after cooling. The surface of the bread wrinkles, and there is a characteristic aroma of extract, a slightly greenish-brown color.	Same as day 1	Same as day 1	Color fades	The growth of mold is almost the same as in Bread C
Bread E	The bread is very fluffy in the oven, but it deflates after cooling. The surface of the bread wrinkles, and there is a characteristic aroma of extract, a slightly greenish-brown color.	Same as day 1	Same as day 1	Color fades	More mold growth than bread D
Bread F	Bread is less fluffy than bread A–E and has a denser texture, brown color, and distinctive aroma.	Same as day 1	Same as day 1	Same as day 1	Same as day 1
Jam A	Pineapple's distinctive yellow color, sharp taste, and distinctive aroma	Same as day 1	Growing mold	Growing mold	Mold grows more and more
Jam B	The yellow color is typical of pineapple, and the typical aroma of pineapple is weak.	Same as day 1	Same as day 1	Growing mold	Mold grows more and more
Jam C	Greenish-yellow color, weak pineapple aroma, and extract aroma	Same as day 1	Same as day 1	Growing mold	Mold grows more and more
Jam D	Pineapple's distinctive yellow color and its distinctive aroma	Same as day 1	Same as day 1	Same as day 1	Same as day 1
Juice A	Bright pink color, more stable juice dispersion, signature aroma of guava juice	Same as day 1	Same as day 1	Formed two layers	Built-in 2 layers

Juice B	Faded pink color, foamy, unstable juice disperses, and a characteristic aroma of guava juice.	Same as day 1	Formed two layers	Formed two layers	Formed two layers
Juice C	Faded pink color, foamy, unstable juice disperses, and a characteristic aroma of guava juice.	Same as day 1	Same as day 1	Same as day 1	Same as day 1
Juice D	Pink color, less stable juice disperses	Formed two layers	Formed two layers	Formed two layers	Formed two layers

a)



b)

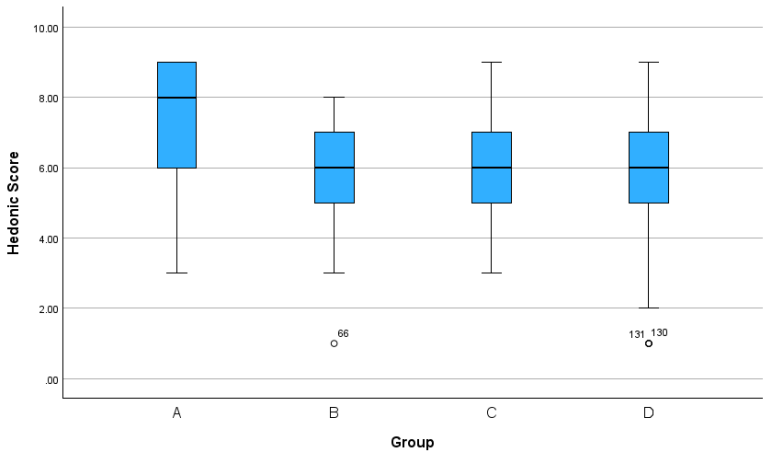
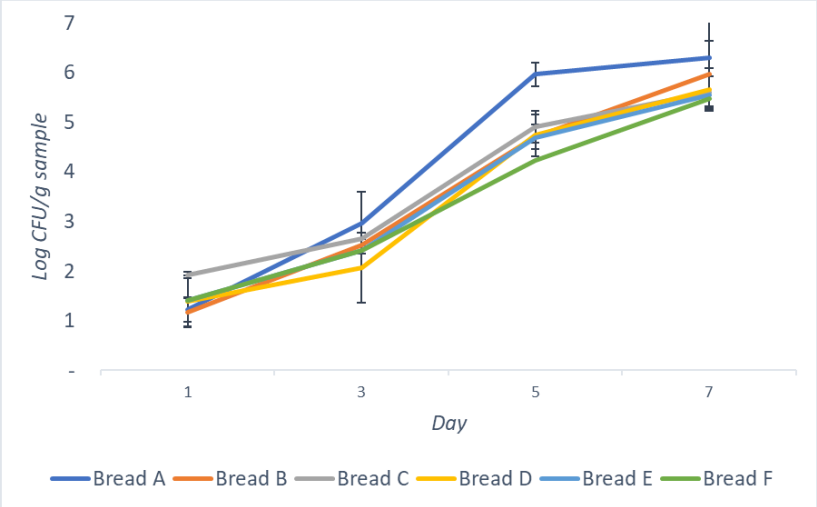


Fig. 1. Hedonic scores for: a) bread samples, and b) juice samples, without preservatives, and with decolorized (DE) and undecolorized (EE) ethanol extracts and potassium sorbate during storage. The values are expressed as mean±standard deviations

a)



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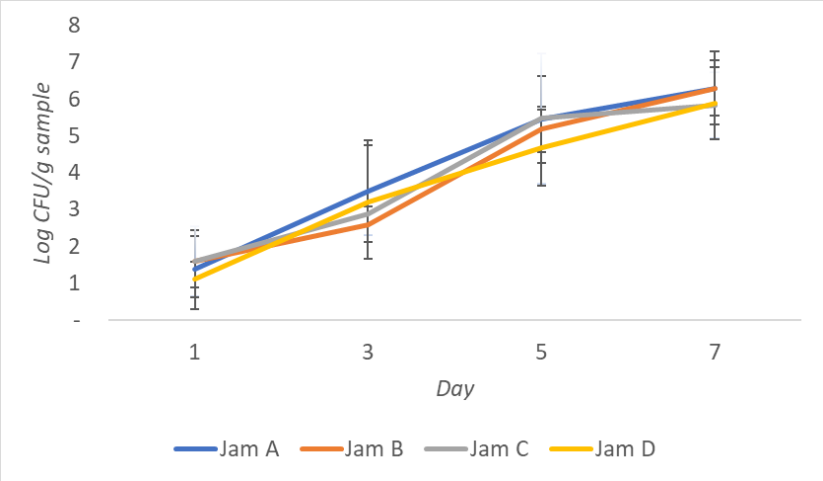


c)

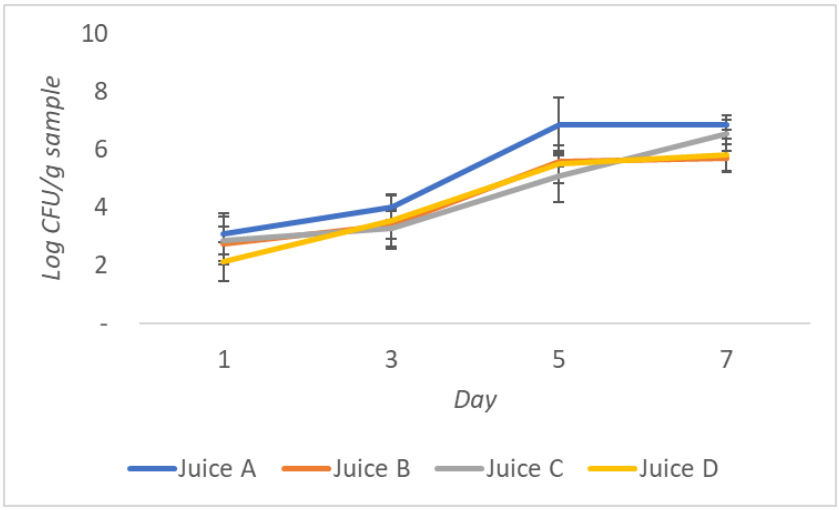
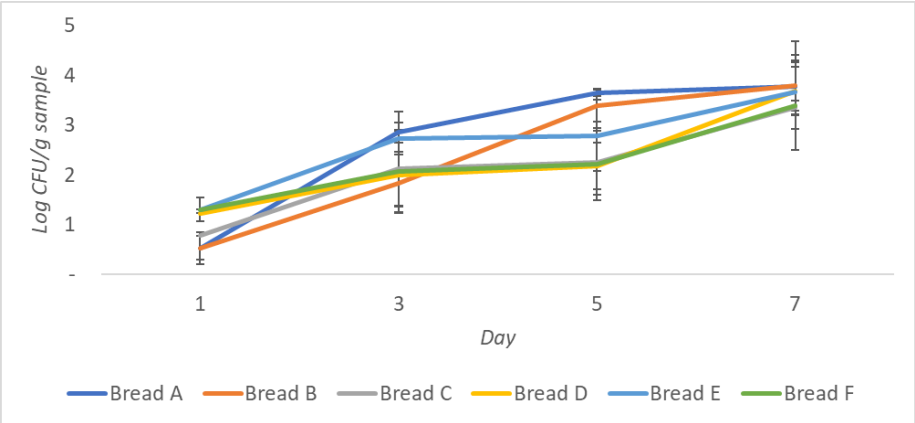
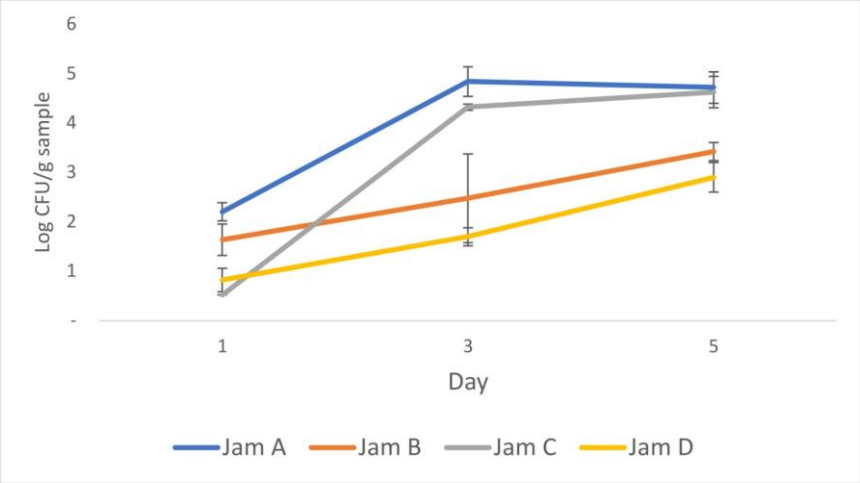


Fig. 2. Total plate count in: a) bread, b) jam, and c) juice samples without preservatives, and with decolorized (DE) and undecolorized (EE) ethanol extracts and potassium sorbate during storage. The values are expressed as mean±standard deviations

a)



b)



c)

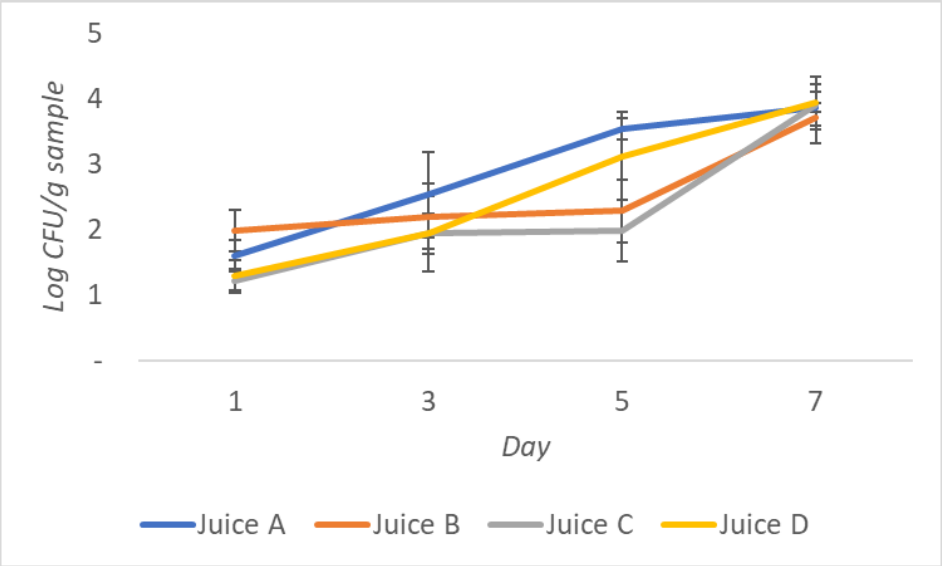
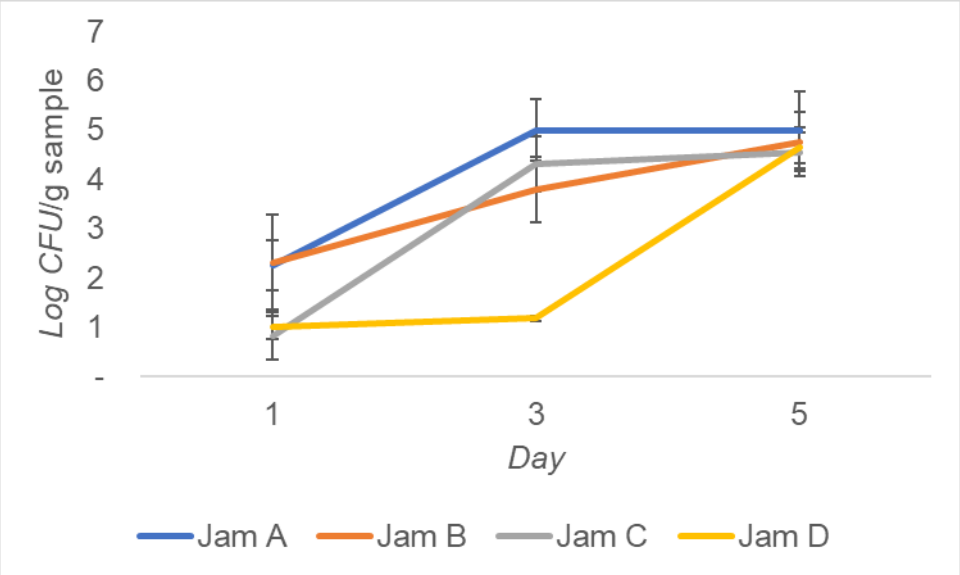


Fig. 3. Yeast number in: a) bread, b) jam, and c) juice samples without preservatives, and with decolorized (DE) and undecolorized (EE) ethanol extracts and potassium sorbate during storage. The values are expressed as mean±standard deviations

a)

b)



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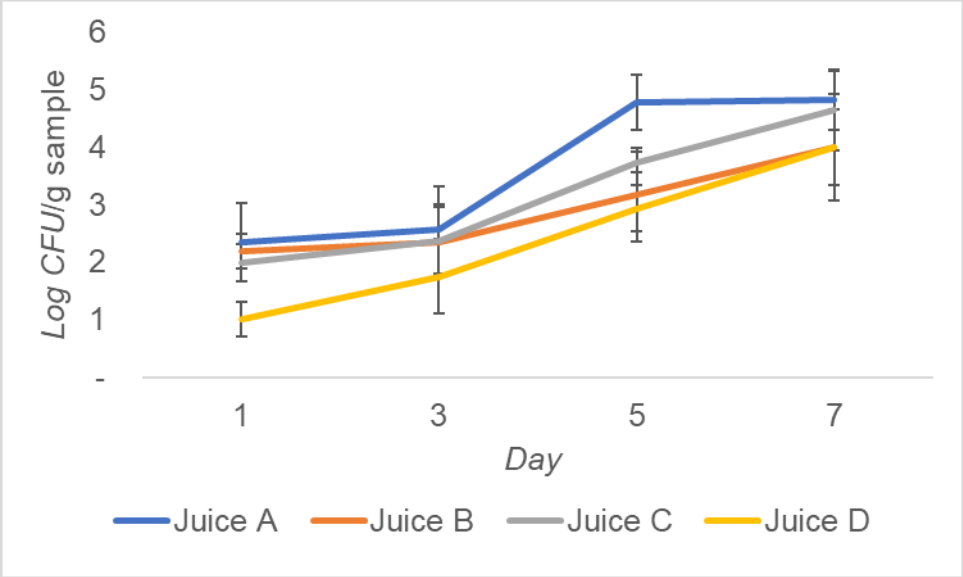

Fig. 4. Mold number in: a) bread, b) jam, and c) juice samples without preservatives, and with decolorized (DE) and undecolorized (EE) ethanol extracts and potassium sorbate during storage. The values are expressed as mean±standard deviations