Food safety assessment of burger patties with added herbal plant material

The microbial status of Wild Garlic and Wild Garlic extracts as ingredients in burger patties

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Burger patties containing Wild Garlic (WG) leaves or Wild Garlic extracts (WGE) were analyzed to determine possible bacterial contamination origination from the plant material. Microbiological analysis of the bacterial load was performed using nine standard ISO methods. In the WG and WGE samples, the presence of *Salmonella* spp., *Listeria monocytogenes,* and Shiga toxin-producing *Escherichia coli* (STEC) was not confirmed. The number of *Escherichia coli* (β-glucuronidase positive), *Enterobacteriaceae,* coagulase-positive staphylococci, and *Bacillus cereus* was < 10 CFU/g. The total bacteria count in the WG sample was within tolerance values (0.04×10^5 CFU/g), but in the WGE, it exceeded the limits set by the WHO (1.10×10^5 CFU/g vs. $> 10^5$). Using the BD BBLCRISTAL Gram-Positive (GP) Identification (ID) system, we identified *Staphylococcus epidermidis, Bacillus pumilus,* and *Staphylococcus aureus*. A particular danger lies in the detected presence of the potential enterotoxigenic and antibiotic-resistant strains of *Staphylococcus aureus,* which was not coagulase positive. The samples of control and fortified BU with WGE were tested on days 0, 5, and 10 during cold storage and 90 days after freezing. By comparing the contamination levels on days 5 and 10, mass contamination was observed in the samples of the modified BU. On day 10, the contamination level was higher compared to day 5. The microbiological load was highest in samples from PB II on both days. The bacterial load of the frozen BU samples was significantly lower after 90 days than during cooling but was still higher than in WGE-fortified BU compared to the control (fully correlated with the amount of WGE added).

KEYWORDS

- *Allium ursinum* L. (Wild garlic)
- Freshly squeezed extract
- Burger
- Microbial contamination

Allium ursinum L. is a member of the *Liliaceae* family. It is called European Wild Garlic (WG), with a number of synonyms (ramson, wood garlic, or bear's garlic) and was called the "Medicinal Plant of the Year" – a title given by the Association for the Protection and Research on European Medicinal plants in 1992 (Sharma et al., 2013). Local cuisines in Europe use WG leaves fresh or dry due to their unique flavor as food seasoning (Radulović et al., 2015). Fresh leaves can be consumed raw or cooked, prepared as a pesto, or added to soups and other dishes. It is also used to flavor hard cheeses or spreads based on cottage cheeses (Janeczko and Sobolewska, 1995; Błazewicz-Wozniak and Michowska, 2011; Sobolewska et al., 2015).

The most important pharmacological activities of WG are its antioxidant, antiplatelet, cardioprotective, cytostatic, antimicrobial, and anti-inflammatory potential (Ivanova et al., 2009; Sapunjieva et al., 2012; Njue et al., 2014; Sobolewska et al., 2015; Liu et al., 2017; Tomšik et al., 2017; Mladenović et al., 2020). Bagiu et al. (2012) reported that allicin is the most important and active substance detected in WG leaves extract. Gobevac et al. (2008) emphasized that the most represented fractions among 20 detected compounds in the volatile oil of *A. ursinum* L. were disulfides (54.7%), followed by trisulfides (37.0%), tetrasulfides (4.7%), and non-sulfur components (1.0%). The study on the content of phenolic acids in fresh WG leaves and bulbs picked in a forest in Western Serbia revealed some differences between free and bound compounds in these plant parts (Djurdjevic et al., 2004). The wide range of biological activities, which enable the presence of valuable bioactive compounds, makes it suitable for the development of various extracts, as well as for creating innovative health-promoting products. The use of WGE as a preservative and antioxidant for food is possible due to its low toxicity (Lee et al., 2020). Considering the value of "clean label" food, we confirmed that consumers recognize ingredients characterized as a "known-natural-good" vs. the opposite (Aschemann-Witzel et al. 2019). Tests of natural sources for phytochemicals and their primarily microbiological safety issues and toxicity have been performed by several authors (Fsanz, 2008; Negi, 2012; Kłębukowska et al., 2013; Van Vuuren et al., 2014; Machado-Moreira et al., 2019; Moore et al., 2019; Bhosale and Padmanabhan, 2021). The demand for herbal medicines is growing significantly as they are used as dietary supplements and for their therapeutic value (Bhosale and Padmanabhan, 2021). Kneifel et al. (2002) pointed out the importance of groups of microorganisms commonly tested in microbiological controls of aboveground (herbal) parts of medicinal plants. Using various plant extracts to prevent meat spoilage and food-borne pathogen activity requires the evaluation of efficacy within meat products or in model systems that closely simulate meat composition (Glass and Johnson, 2004; Sospedra et al., 2010; Klimešová et al., 2015). The objective of this study was to examine food safety and public health aspects of WGE usage in burgers as a food matrix: a) evaluation of microbial load in raw plant material and freshly squeezed WGE; b) precise determination of the causative agents of contamination from WGE; c) examination of the effects of modifying burgers with WGE, which could potentially be a source for contamination.

Materials and methods

Plant material

The *Allium ursinum* L. (WG) was collected in a protected area (The Ovčar-Kablar Gorge, latitude 43°54'02.8", longitude 20°11'54.7" and 391 m altitude) in March 2020. The landscape is far from any polluters. Hand-picked WG leaves were frozen at –80 °C prior to analyses. A voucher specimen was deposited in the Herbarium of the Institute of Botany and Botanical Garden Jevremovac, University of Belgrade – number 17817 BEOU (Thiers, 2021).

Preparation of wild garlic extracts (WGE)

The freshly squeezed extract was obtained by squeezing WG leaves using a manual mini berry squeezing press. This approach was chosen to minimize the risk of loss of bioactive substances. The pH value of raw (non-filtrated) WGE was 6.50.

Burger (BU) preparation

The BUs were prepared in a registered company with its own plant for processing low volumes of meat in accordance with good hygienic and manufacturing practices. Four production batches (PB) of BU were prepared in triplicate. All formulations of BU were made with 50% pork shoulder, 20% beef shoulder and neck, 15% fatty beef trimmings with 30% fat, 10% water, 2% table salt, 0.5% white pepper, 0.8% ground sweet red pepper, and 1%

Received: 25 January 2022 | reviewed: 1 August 2022 | revised: 4 September 2022 | accepted: 23 September 2022

Pergeta cooking supplement with vegetables. Three modified batches were produced by directly adding WGE into BU, and applied in various effective concentrations (1.32 mL/kg of minced meat for BU production in PB I, 4.40 mL/kg of WGE in PB II, and 8.79 mL/kg in PB III). The meat was minced in a meat grinder (Sind, Šabac, Serbia) to about 14 mm, hand salted by dry process, and subjected to ripening for approximately 12 hours. Additives and spices were mixed with minced meat in a blender (Fimar – Villa Verucchio – Rimini – Italy, Model IC50CIP40050T, serial No. 130900591, 40 V, 1500 W, 50 Hz, IPX3, 2013) for 5 minutes. The second grind was to a diameter of 5.8 mm. Each BU was prepared with 120 g of minced meat and shaped in a manual molding press. BUs were packed in polystyrene packaging (9–12 burgers in one food container) and clearly labeled. Microbiological analyses were performed during cold storage at 4 °C (day 0, 5, and 10) and after freezing at –20 °C (on day 90 after preparation).

Determining the presence of bacterial contamination – microbiological safety of WGE

The authors performed microbiological tests of WG leaves and WGE for the presence of bacterial contamination, using standard ISO methods:

- SRPS EN ISO 6579-1:2017/A1:2020 – Microbiology of the food chain – Horizontal method for the detection, enumeration, and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp.;
- SRPS EN ISO 11290-1:2017 – Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and *Listeria* spp. – Part 1: Detection method;
- SRPS EN ISO 21528-2:2017 – Microbiology of the food chain – Horizontal method for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count technique;
- SRPS EN ISO 6888-1:2009 – Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 1: Technique using Baird-Parker agar medium;
- SRPS EN ISO 4833-1:2014 – Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 1: Colony count at 30 degrees C by the pour plate technique;
- SRPS EN ISO 7932:2009 – Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of presumptive *Bacillus cereus* – Colony-count technique at 30 degrees C;
- SRPS CEN ISO/TS 13136:2014; Microbiology of food and animal feed – Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens – Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups (ISO/TS 13136:2012).

If the presence of bacteria was determined, their identification was performed. Samples were prepared according to the manufacturer's instructions, in this case, BD BBL CRISTAL Gram-Positive (GP) Identification (ID) system (Becton, Dickinson, and Company, USA – BD 245240), designed to identify aerobic gram-positive bacteria.

To determine the microbial quality of the conventional and fortified (with WGE) BU as a food matrix, the following three methods were performed:

- SRPS EN ISO 6579-1:2017/A1:2020 - Microbiology of the food chain - Horizontal method for the detection, enumeration, and serotyping of *Salmonella* - Part 1: Detection of *Salmonella* spp.;
- SRPS ISO 16649-2:2008 - Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* - Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl β-D-glucuronide;
- SRPS EN ISO 4833-1:2014 - Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 °C by the pour plate technique.

Above mentioned methods are following the Law on Food Safety (Official Gazette No. 41/2009) and corresponding by-laws and food safety reports, as well as according to European regulations, e.g., Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

Decontamination of WGE (after contamination was determined) was done by microbiological filtration. The crude WGE was filtered through a microbial filter with pores of 0.45 mm (Filtropur S 0.45, Lot 90245103, Sterile, SARSTED AG & Co. KG, Sarstedstr. 1, D-51588 Nümbrecht, Germany), followed by an examination according to SRPS EN ISO 4833-1:2014.

All of the analyses mentioned above were performed at the Department for Testing of Raw Materials of Animal Origin, Food and Water (Sector for Laboratory Testing) at the Veterinary Specialist Institute Kraljevo, in Kraljevo, Serbia.

Statistical analysis

The microbiological analyses were carried out in triplicates, and the results were subjected to two-way analysis of variance (ANOVA), which was used to evaluate the effect of extract addition, storage time (cooling & freezing) and its interaction. Statistica 12.5 (StatSoft, Inc., Tulsa, OK, USA) software was used to perform statistical analysis. Differences between means were determined using Tukey's HSD test at the significance level of $p < 0.05$ (95% confidence interval).

Results and discussion

The main reason for designing our study was for the adequate education of consumers, for whom "natural" and "harmless" are synonyms, although the opinion of experts is diametrically opposed. The thesis that the plant extracts are not *a priori* safe, and that protocols must be devised to control their contamination, primarily microbiological, is supported by the results of our research. The authors identified the types of bacteria that are contaminants of WG and WGE while being important for food safety issues and determined their nature (pathogenic or non-pathogenic). In their study, the scientists examined the effect of modifying BU with WGE, which could potentially be a contaminant in processed meat products, despite complicated interactions (antimicrobial and antioxidant effects) in the complex matrix, which contains a number of different substances that protect against microorganisms.

After we got acquainted with the characteristics of the identified bacteria (Tab. 1), ● the authors successfully removed them from the raw material by microbiological filtration, as discussed below, so that they would not be sources of contamination when seasoning or preserving food.

The results of the bacterial determination of contaminated WG leaves and WGE are presented in Table 1. The first microorganism isolated from WGE was *Staphylococcus* spp. ($> 0.15 \times 10^5$ CFU/g), which is not coagulase positive. By further identification, using the BD BBL CRISTAL Gram-Positive (GP) Identification (ID) system, we found that the tested microorganism is a representative of the genus *Staphylococcus* spp. in fact, *Staphylococcus aureus* (*S. aureus*), although it was not coagulase positive. In routine laboratory practice, the production of coagulase is the only criterion to distinguish *S. aureus* from other staphylococci. It is also important to note that coagulase-negative strains of *S. aureus* have been reported (Vandenesch et al., 1994). An example is the *S. aureus* reference strain Newman D2C, which is coagulase-negative and non-haemolytic. There is evidence that coagulase-negative *S. aureus* may even be methicillin-resistant *Staphylococcus aureus* (MRSA) (Olver, 2005; Bayston, 2006). WHO reported that in some regions of Africa, as many as 80% of *S. aureus* infections are provoked by MRSA strains, because treatment with a new generation of antibiotics was ineffective (WHO, 2014). *Staphylococcal* enterotoxins are a major cause of food poisoning, which typically occurs after ingesting processed meat and dairy products, contaminated with *S. aureus* by unhygienic handling and subsequent storage at higher temperatures (Katić, 2008; Hennekinne et al., 2012). Coagulase-negative staphylococci (CoNS) currently include over 30 species, 15 of which are human pathogens (one of them being *Staphylococcus epidermidis* which was identified in this study, Tab. 1). While *S. epidermidis* is not usually pathogenic, patients with compromised immune systems are at risk of developing an infection. As part of the normal skin flora, *S. epidermidis* is a frequent contaminant of specimens sent to the diagnostic laboratory (Queck and Otto, 2008). A special danger was described by Brown and Jiang (2008), which isolated antibiotic-resistant bacterial species (*Bacillus* spp., *Erwinia* spp., *Ewingella americana*, *Staphylococcus* spp., *Enterobacter cloacae*, and *Stenotrophomonas maltophilia*) in 29 herbal supplements commercially sourced in local US stores. The use of herbal medicines, which are sold

Analyzed bacteria

Tab. 1: Microbiological analysis of Wild garlic leaves (WG) and Wild garlic extracts (WGE)
Tab. 1: Mikrobiologische Analyse von Bärlauch (WG) und Bärlauchextrakten (WGE)

Parameters	Method	WG cfu/g	WGE cfu/g	Found contaminants
Salmonella spp.	SRPS EN ISO 6579-1:2017	Not detected in 25 g	Not detected in 25 g	–
Listeria monocytogenes	SRPS EN ISO 11290-1:2017	Not detected in 25 g	Not detected in 25 g	–
Escherichia coli (β-glucuronidase positive)	SRPS ISO 16649-2:2008	< 10 cfu/g	< 10 cfu/g	–
Enterobacteriaceae	SRPS ISO 21528-2:2017	< 10 cfu/g	< 10 cfu/g	–
Coagulase positive staphylococci	SRPS EN ISO 6888-1:2009	< 10 cfu/g	< 10 cfu/g (> 0.15 × 10^5 cfu/g *coagulase negative Staphylococcus* spp.)	*S. aureus* - coagulase negative *S. epidermidis*
Aerobic colony count	SRPS EN ISO 4833-1:2014	0.04 × 10^5 cfu/g	1.10 × 10^5 cfu/g	*S. aureus* - coagulase negative *S. epidermidis* *Bacillus pumilis*
Bacillus cereus	SRPS EN ISO 7932:2009	< 10 cfu/g	< 10 cfu/g	–
Shiga toxin-producing *Escherichia coli* (STEC)	SRPS CEN ISO/TS 13136:2014	Not detected in 25 g	Not detected in 25 g	–

Source: Kurćubić et al.

uncontrolled due to lack of regulations can be risky for the health of consumers, if the medicines are contaminated with pathogenic bacteria resistant to antibacterial drugs, because infected persons are harder and more challenging to treat (Kira et al., 2021). Shamsuddeen (2009) determined the high values for the presence of *Staphylococci* (geometric mean was 1.73 × 10^9 CFU/g) in the spice mixture (ginger, cloves, black pepper, groundnut, salt and seasoning), but he didn't specify the staphylococcal species. The total number of bacteria in the leaf was within tolerance limits (0.04 × 10^5 CFU/g). In the WG extract, the number of microorganisms exceeded the limits set by the WHO: 1.10 × 10^5 CFU/g vs. >10^5 CFU/g microorganisms (WHO, 2007). *Salmonella* spp., *Listeria monocytogenes* and Shiga toxin-producing *Escherichia coli* (STEC) were not detected in 25 g of samples, whether WG leaf samples or WGE. The number of *Escherichia coli* (β-glucuronidase positive), *Enterobacteriaceae*, *Staphylococcus aureus* and *Bacillus cereus* was < 10 CFU/g in the WG and WGE. In subsequent identification using the BD BBL CRISTAL GP, we discovered *Bacillus pumilus*, a Gram-positive, rod-shaped and resistant endospore-forming bacterium, which is a common cause of food spoilage and in some cases foodborne poisoning and human infections, e.g. anthrax-like cutaneous lesions (Branquinho et al., 2014). There is a similarity between the results of our study and the study of Noor et al. (2013), conducted in Bangladesh, who examined 85 liquid samples and found that 2 samples showed very high bacterial load (1.24 × 10^5 CFU/mL or CFU/g), and one sample showed contamination by coliforms (indicator of poor hygiene, i.e., faecal contamination). None of the tested samples showed the presence of *Salmonella* spp. and *Shigella* spp.

According to modern requirements for the application of hygiene standards, so-called "attributive class plans" usually prescribe a unique strategy for estimating and defining the number of test samples, which must have a certain mass to ensure the safety and accuracy of microbiological analysis. The above plans allow for descriptive conclusions about the microbiological quality of the tested samples ("good" vs "bad" or "less than" vs "more than", "acceptable" vs "unacceptable") and the set risk assessment must provide for a clear distinction between accepted and rejected production batches of natural products. In the case of medicinal plants, the presence of classical pathogens (*Salmonella* spp., *Listeria monocytogenes*, EHEC and *Campylobacter*) is not tolerated in any of the tested samples. Therefore, standardized microbiological methods test samples weighing 10 or 25 grams or more (depending on the degree of risk). The only acceptable result obtained by the described analyses should be described as "not detected in ... grams" (as in this study). Hence, the plans of the two classes represent a very strict strategy. The "3 class plans" offer compromise solutions in the evaluation of tested natural products, as a more flexible difference is defined between the levels of microbiological quality of "acceptable", "still acceptable or tolerated" and "unacceptable" samples (Kneifel et al., 2002).

It is believed that the most common pollutants are harmless and may have originated in the places where the plants were harvested. Some of the potentially harmful pathogens identified in the samples may indicate unhygienic conditions and methods of handling and processing plant products (Van Vuuren et al., 2014).

The results of the determination of the microbial quality of BU modified by WGE, obtained by applying the three methods presented in the section on material and methods, are given in Tables 2 and 3. ●

After the synthesis and analysis, serious microbiological contamination of WGE was observed, which instead of the expected antimicrobial effect caused the contamination of BU modified with different concentrations of WGE. On day 0 of BU production, the contamination was higher in the samples of the control group (C – conventional, without added WGE), compared to the modified batches of BU (PB I, PB II and PB III). Comparing the levels of contamination on days 5 and 10 during cold storage, mass contamination was observed in the samples of the modified BU, with a higher level on day 10 compared to day 5. The microbiological load was highest in BU samples from PS II on both days. Note that the amount of added extract was in line with previous results (PATENT APPLICATION PUBLICATION PUB. NO.: US 2007/0160725 A1, 2007, United States), compared to other ingredients (minced meat) of extremely small amounts (1.32 mL/kg, 4.40 mL/kg and 8.79 mL/kg, respectively).

Bacterial contamination after 90 days of freezing was significantly lower than during cooling but was still higher in WGE-fortified batches (fully correlated with the amount of WGE added).

Detection analysis did not reveal the presence of *Salmonella* spp. and *E. coli* in samples of both control BU and modified BU, throughout the duration of the experiment. Using the method SRPS EN ISO 4833-1: 2014, the detection of the number of microorganisms in food (BU samples) was performed. On day zero, we observed that the total number of microorganisms was lower in the samples of experimental groups compared to the control. A higher number of microorganisms in the WGE-enriched samples than in the control group was observed on days 5 and 10 of refrigerated storage and on day 90 after freezing.

Statistically significant differences between the determined values for the number of microorganisms in BU samples, stored at 4 °C, were found in the samples of the control group between days 0 and 5. Differences in the

number of microorganisms in the samples of modified BU (PB I, PB II and PB III), observed between days 0, 5 and 10 of cold storage, were also statistically significant. Non-significant differences were identified between all observed treatments (tmt = C or PB) and interactions of treatments (production batches – PB) with a term for sample testing (day of examination).

Microbiological examinations of BU samples after 90 days of freezing revealed statistically significant differences between samples of the control and PB III experimental group ($p < 0.05$). A comparison considering the effect of frozen storage (day 0 vs. day 90) revealed that there were statistically significant differences within all experimental groups.

According to WHO guidelines (WHO, 2007) for assessing the quality of herbal medicines with reference to contaminants and residues, the colony counts should not exceed 10^5 CFU/g for medicinal plant materials intended for internal use. For plant products intended for topical use or those pretreated with boiling water, the colony count should not exceed 10^7 CFU/g (WHO, 2007). Similar limits for bacterial contamination of herbal medicines are given in the European Pharmacopoeia for total aerobic bacteria (10^5 CFU/g), yeasts, moulds, *Enterobacteria* and other Gram-negative organisms (10^3 CFU/g) and *E. coli* and *Salmonella* (should be absent), as reported by Okunlola et al. (2007). Although many of the plant materials are below admissible limits, what appears important to understand is that in many cases, these organisms accumulate in the extract and then the extract will be loaded with microbes which ultimately will not be suitable for human use. Our study was comparable to other studies on the microbial load of herb extracts as a serious health hazard. It is of great importance for researchers and scientists to be familiar with environmental pollutants and their effects when publishing research on the activity and efficacy of raw plant extracts (Street et al., 2008). Specific microbial contamination in plants may adversely affect the concentration of active ingredients (Agarwal et al., 2014). Accurate assessment of the microbial load in the raw material itself is a key factor in achieving cleaner plant extracts that will show maximum therapeutic potential. In this study, we proved that the tested unprocessed WGE became a serious source of contamination for modified PB of BU.

Proliferation rate

Tab. 2: Contamination load of Wild garlic extracts-modified burger samples after 0, 5, 10 and 90 days of storage
Tab. 2: Der Kontaminationsgrad von mit Bärlauchextrakt angereicherten Burgerproben nach 0, 5, 10 und 90 Tagen Lagerung

Duration	Method	Parameters	Unit of measure		C	PB I	PB II	PB III
0*	SRPS EN ISO 6579/1:2017	*Salmonella* spp.	in 10 g		not detected			
	SRPS ISO 16649-2:2008	*E. coli*	cfu/g		< 10			
	SRPS EN ISO 4833-1:2014	number of microorganisms at 30 °C	cfu/g	mean	**0.18×10^5**	**0.09×10^5**	**0.03×10^5**	**0.05×10^4**
5	SRPS EN ISO 6579/1:2017	*Salmonella* spp.	in 10 g		not detected			
	SRPS ISO 16649-2:2008	*E. coli*	cfu/g		< 10			
	SRPS EN ISO 4833-1:2014	number of microorganisms at 30 °C	cfu/g	mean	**5.73×10^5**	**5.80×10^5**	**9.10×10^5**	**8.33×10^5**
10	SRPS EN ISO 6579/1:2017	*Salmonella* spp.	in 10 g		not detected			
	SRPS ISO 16649-2:2008	*E. coli*	cfu/g		< 10			
	SRPS EN ISO 4833-1:2014	number of microorganisms at 30 °C	cfu/g	mean	**133.33×10^5**	**153.33×10^5**	**181.67×10^5**	**143.33×10^5**
90	SRPS EN ISO 6579/1:2017	*Salmonella* spp.	in 10 g		not detected			
	SRPS ISO 16649-2:2008	*E. coli*	cfu/g		< 10			
	SRPS EN ISO 4833-1:2014	number of microorganisms at 30 °C	cfu/g	mean	**0.40×10^5**	**0.56×10^5**	**0.75×10^5**	0.84×10^5

* cold storage at 4 °C, ** frozen storage at –20 °C

Source: Kurćubić et al.

Microbial load

Tab. 3: Bacterial count (log cfu/g) in control and modified burger samples
Tab. 3: Keimzahl (log KbE/g) in Kontroll- und modifizierten Burgerproben

Duration	C	PB I	PB II	PB III	Significance (p)		
		cold storage			tmt	time	tmt*time
0	4.05±0.58^{A}	3.88±0.27^{A}	3.47±0.07^{A}	3.63±0.30^{A}	NS	0.000	NS
5	5.75±0.11^{B}	5.75±0.12^{B}	5.95±0.09^{B}	5.92±0.05^{B}			
10	7.12±0.08^{B}	7.18±0.10^{C}	7.26±0.02^{C}	7.15±0.08^{C}			
		frozen storage					
0	4.05±0.58	3.88±0.27^{A}	3.47±0.07^{A}	3.63±0.30^{A}	NS	0.000	0.0485
90	4.59±0.16^{a}	4.73±0.15^{abB}	4.87±0.08^{abB}	4.92±0.09^{bB}			

tmt: treatment; NS: not significant.
$^{a-b}$ Values (mean±SD) in the same row with different superscripts are significantly different ($p<0.05$).
$^{A-C}$ Uppercase letters are used for comparing the samples considering the effect of storage.
Values in the same column for the same property, with different superscripts, are significantly different ($p<0.05$).

Source: Kurćubić et al.

As for how certain bacteria can be removed from WGE, the answer offered by this study is: by microbiological filtration method. On three occasions, crude WGE after confirmed contamination was filtered through a microbiological filter with pores of 0.45 mm (Filtropur S 0.45, Lot 90245103, Sterile, SARSTED AG & Co. KG, Sarstedstr. 1, D-51588 Nümbrecht, Germany). After filtering, WGE was examined by SRPS EN ISO 4833-1:2014. All three times the medium remained sterile (0 CFU/mL), which proved that filtration was a simple, cheap, and successful method of removing undesirable microorganisms from plant extracts.

De Sousa Lima et al. (2020) researched the microbial quality (isolation and identification of pathogenic bacteria) of commercial and homemade herbal medicines. Pathogenic bacterial isolates were preliminarily characterized by colony morphology, Gram staining, and biochemical tests (oxidase, gas and catalase production), at the end of the incubation period. Among the 132 herbal products (HP) analyzed, only 22% of the homemade HP were prepared with a single plant species. A total of 31.8% of tested samples exceeded the safety limits (CFU/g ≤ 10^5) according to WHO guidelines (WHO, 2007) for aerobic bacteria, including 16.7% of the homemade HP and 15.1% of the commercial HP, indicating risk in the consumption of the analyzed products.

Additional confirmation of the validity of the results of our research and the motivation to deepen it lies in the fact that liquid preparations of herbal medicines, which are given orally, are most often contaminated. When the differences in contamination between pathogenic bacteria (qualitative test) were significantly different (p = 0.005/chi-square), it was demonstrated that homemade herbal medicines had a higher risk of contamination by pathogenic bacteria than commercial herbal medicines (De Sousa Lima et al., 2020), which further strengthens our belief that our research is completely justified. In the same study, the authors announced that the most commonly isolated bacteria from herbal medicines include *S. aureus* (49.2%), *Salmonella* spp. (34.8%), *E. coli* (25.8%), and *P. aeruginosa* (14.4%), making them inappropriate for consumption. Herbal medicines are the cheapest way of treatment for various diseases, as they can be easily prepared and bought over the counter and outside of pharmacies. It is quite true that the integration of herbal medicines into the primary health care system of developing countries is expanding. For this very reason, the safety issues of natural sources of bioactive substances must not be neglected. The recommendation of the Wirtschaftsvereinigung Kräuter und Früchtetee e.V., a member of the European Herbal Infusions Association, can be considered as one of the potential solutions for maintaining the safety of plant products: a note should be printed on tea-boxes cautioning the consumer: "always boil the water and allow to infuse for at least 5 minutes to ensure the safety of use" (Kolb, 1999).

Conclusions

This study is one of few reports for assessing the levels of microbial contamination of WG leaves and WGE, with an assessment of the impact of its various concentrations on the microbiological safety of BU, due to complicated incompatibility problems between plant products and the food constituents. It is necessary to raise awareness about microbial hazards of spices and medicinal herbs, without causing alarm or damaging consumer confidence in these foods. It is important to develop and improve knowledge and skills in good hygienic and good manufacturing practices. Recommendations on good agricultural and sourcing practices in the EU and WHO guidelines for raw herbal materials provide the basis for appropriate quality assurance. Beyond compliance, following current national and international legislation is important. It is also necessary to monitor all stakeholders who prepare and market herbal preparations, checking whether they have a license from the Ministry of Health and whether the products are registered and authorized for human use, because there are known cases of counterfeiting and fraud. Campaigns initiated by ministries (Ministry of Health and Ministry of Agriculture) would be essential, connecting primary health care units, agricultural professional services, scientific institutes and faculties, as well as national centres of excellence, with the aim of bringing together all producers and sellers. Finally, based on the significant and illustrative results reported in our study, which relate to the contamination status of WG, WGE and BU enriched with unfiltered WGE, we dare to recommend that continuous monitoring and improvement of microbiological standards be urgently introduced for natural herbal remedies and food additives produced from them, available in the market.

Practical importance

Our research is one of the few reports to assess the levels of microbial load of Wild Garlic leaves (WG) and Wild Garlic extracts (WGE) and their impact on the microbiological safety of burgers (BU). The accurate assessment of the plant's microbial load is a key factor in achieving safer extracts that will also show maximum therapeutic potential. In our study, we proved that the tested unprocessed freshly squeezed WGE became a serious source of contamination for modified PB of BU. We made simple, practical and proven recommendations for the processing of plant extracts as a medicinal product or to fortify various foods, without food safety issues. On three occasions, crude WGE (proven contaminated) was poured through a filter with pores of 0.45 mm. After filtering, WGE was examined according to SRPS EN ISO 4833-1:2014 method. All three times the medium remained sterile, which showed that filtration was a simple, cheap and successful method of removing undesirable microorganisms from plant extracts. It is necessary to monitor all manufacturers and distributors of market herbal preparations. Our research contributes to the initiative to connect food business operators and national food safety authorities to synergistically lay the ground for creating safe products for heightened consumer food safety.

Acknowledgements

The work is part of the research project record number 046009 III – Annex to the contract Ref. No. 451-03-68/2022-14/200088, funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Zusammenfassung

Bestimmung der Lebensmittelsicherheit von Burgerpattys mit Kräuterzusatz

Die Keimbelastung von Bärlauch und Bärlauchextrakten als Zutat in Burgerpattys

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Allium ursinum L. (Bärlauch) | Frisch gepresster Extrakt | Burger | Mikrobielle Kontamination

Burgerpattys mit Bärlauch (WG) oder Bärlauchextrakt (WGE) wurden analysiert, um das Risiko einer bakteriellen Verunreinigung durch den pflanzlichen Rohstoff zu ermitteln. Die mikrobiologische Analyse der bakteriellen Belastung wurde mit neun ISO-Standardmethoden durchgeführt. In den WG- und WGE-Proben konnte keine Kontamination durch *Salmonella* spp., *Listeria monocytogenes* und Shiga-Toxin produzierenden *Escherichia coli* (STEC) festgestellt werden. Die Anzahl von *Escherichia coli* (β-Glucuronidase-positiv), *Enterobacteriaceae*, koagulase-positiven Staphylokokken und *Bacillus cereus* lag bei < 10 KbE/g. Die Gesamtkeimzahl in der WG-Probe lag innerhalb der Toleranzwerte (0,04 × 10^5 KbE/g), aber in der Probe mit Bärlauchextrakt überschritt sie die von der WHO festgelegten Grenzwerte (1,10 × 10^5 KbE/g vs. > 10^5). Mit dem BD BBLCRISTAL Gram-Positive (GP) Identifikationssystem (ID) wurde die Kontamination mit *Staphylococcus epidermidis*, *Bacillus pumilus* und *Staphylococcus aureus* nachgewiesen. Eine besondere Gefahr liegt in der Präsenz der potenziell enterotoxischen und antibiotikaresistenten Stämme von *Staphylococcus aureus*, die nicht koagulasepositiv waren. Die Kontrollproben und die mit Bärlauchextrakt angereicherten Burgerpattys wurden an den Tagen 0, 5 und 10 während der Kühlung und 90 Tage nach dem Einfrieren untersucht. Beim Vergleich der Kontaminationsniveaus an den Tagen 5 und 10 wurde bei den Proben der modifizierten Burgerpattys eine Massenkontamination festgestellt. Am Tag 10 war der Kontaminationsgrad höher als am Tag 5. Die mikrobiologische Belastung war in den Proben aus PB II an beiden Tagen am höchsten. Die bakterielle Belastung der gefrorenen Proben war nach 90 Tagen signifikant niedriger als während der Kühlung, jedoch höher als bei den mit Extrakt angereicherten Pattys verglichen mit der Kontrollprobe (vollständig korreliert mit der Menge des zugesetzten WGE).

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