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# Microbiota Composition in Raw Drinking Milk from Vending Machines: A Case Study in Croatia

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**Abstract:** According to the Regulation on the Quality of Fresh Raw Milk, up to 100,000 microorganisms/mL are allowed in milk obtained by the hygienic milking of healthy cows, which represents the natural microbiota of milk and has no negative impact on the overall quality of milk. However, with unprofessional handling during and after milking, milk is easily contaminated and becomes a potential medium for the growth and reproduction of microorganisms, some of which can be harmful to human health. Since the number of aerobic mesophilic bacteria in milk is one of the indicators of the hygienic quality of milk, their number and identification are fundamental in the control of raw milk from milk vending machines. From five different milk vending machines, 35 samples were collected, from which the total number of aerobic mesophilic bacteria was determined using the flow cytometry method and the classic method of counting colonies on a nutrient medium. Randomly selected colonies based on morphological differences (n = 700) were identified by comparing MALDI-TOF mass spectra with reference spectra stored in the microorganism library and processing using the MALDI Biotyper computer program. Thirty-eight genera and eighty-one bacterial species and five genera and seven fungal species were successfully identified. The species that predominate are *Lactococcus lactis*, *Hafnia alvei*, *Escherichia coli*, *Leuconostoc mesenteroides*, and *Kluyveromyces lactis*. By integrating advanced methods like flow cytometry and MALDI-TOF MS for precise microbial identification, this study highlights the need for enhanced monitoring and adherence to hygienic standards in raw milk vending machines. This approach not only safeguards public health but also supports consumer confidence in milk quality from vending machines.

**Keywords:** raw milk; vending machine; hygiene quality; microbiological hazards



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## 1. Introduction

Raw milk, due to its rich chemical composition, is a very suitable medium for the growth and reproduction of diverse microbial flora, including pathogens transmissible to humans. Despite this, there has been an observed trend in different countries of an increased consumer demand for milk with higher nutritional value and better taste, which has not been subjected to prior thermal processing [1–4]. On the other hand, some countries have banned the consumption of raw milk due to the public health risk related to

foodborne pathogens such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp., enterohaemorhagic *Escherichia coli*, and others [5]. For example, the European Food Safety Authority (EFSA) Scientific Opinion from 2015 reported the results of the Czech Republic study, carried out regarding the rapid expansion of vending machines and increased consumption of raw milk [5]. They evaluated the microbiological and hygienic quality of raw milk from vending machines and confirmed the presence of the pathogens *Staphylococcus aureus*, *Campylobacter* spp., *Salmonella* spp., and *L. monocytogenes* [5].

In any case, milk microbiota can be composed of different types of bacteria that can grow in a wide temperature range. The majority of milk-related pathogens belongs to the mesophilic group and can be part of the microbiome counted during regular testing of the total viable count [6,7]. The total viable count (TVC) and somatic cell count (SCC) are two parameters used in assessing the hygienic quality of raw milk and udder health, with a permitted limit of 100,000 CFU/mL and 400,000 cells, respectively [8].

These requirements apply also to raw cow's milk from vending machines (raw milk that is intended for public consumption without prior heat treatment) in Croatia, ignoring the microbiological hazards possibly present in this type of production and placing on the market [9,10]. However, there is national Guidance for microbiological criteria in food [11] which sets the standards for milk intended to be eaten raw, considering *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *Enterobacteriaceae*, sulphite-reducing clostridia, and aerobic mesophilic bacteria. However, these criteria are only recommended, and the testing for these specific pathogens and contaminant bacteria is not obligatory [12,13]. However, there is an obligation to warn consumers that thermal processing of raw milk from vending machines is recommended before consumption [5].

A low number of microorganisms is naturally present in milk and mainly belong to the genera *Micrococcus* and *Staphylococcus* (30–99%), *Streptococcus*, *Lactococcus*, and *Lactobacillus* (1–50%) and sporadic Gram-negative bacteria or yeasts [6]. A significant increase in the number of microorganisms in milk is primarily the result of animal disease and/or udder infection and the lack of good production and hygiene practices on farms [6,14–16].

Under conditions of hygienically appropriate milking, the initial microbial contamination is $<10^4$ CFU/mL and is considered acceptable for the microbiological quality of raw milk. Conversely, the microbial population in raw milk after milking exceeds $10^6$ CFU/mL under conditions of poor hygiene and/or mastitis [10].

In fact, the contamination of raw milk with pathogenic bacteria, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Escherichia coli*, is common if there is no systematic control of mastitis in the herd. Other less common causes of mastitis include *Corynebacterium pyogenes*, *Mycobacterium bovis*, *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* spp., and coagulase-negative staphylococci [6,17–19]. In addition to this, major pathogens *Brucella* spp. (*B. abortus*, *B. melitensis*) and *Coxiella burnetii*, which cause brucellosis and Q fever upon consuming contaminated raw milk and milk products, are also a serious health concern in poor hygiene environments [20,21]. The majority of listed pathogens are zoonotic and known agents of foodborne infections or intoxications [22]. The contamination of milk with zoonotic pathogenic bacteria may occur from environmental sources including farmers, milking machines, the tanks for milk storage at the farm, or even transport equipment [5]. As an example, there is evidence of the persistence of *L. monocytogenes* in the milk chain, i.e., milk distributed by vending machines in Croatia [12]. In this study, the pathogen prevalence in raw milk correlated well with high TVC in corresponding milk collected at specific vending machines owned by incriminating farms. Its persistence in dairy environments may be related to unique ability to form biofilms on contaminating surfaces and its high resistance to disinfectants [23].

There is the question of whether the consumer can rely on standard testing of TVC and SCC in raw milk distributed further by vending machines in terms of pathogenic threats. Managing the risk of foodborne diseases from the point of the vending machine to the point of consumption is now on the consumer. Based on an HAPIH study [3], 48.6% of consumers of milk from vending machines in Croatia do not boil the milk. Having in mind these facts, the aim of this study was to obtain insight into the microbial population composition from counting aerobic mesophilic bacteria during standard milk testing [24]. The composition of this microbial group is evaluated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), with the assumption of the possible presence of bacterial pathogens in milk from vending machines. In doing so, multivariate analysis tools such as (i) heat maps, (ii) principal component analysis, and (iii) Clustering trees were used.

## 2. Materials and Methods

### *2.1. Milk Sampling*

A total of 35 samples of fresh raw cow's milk were collected from five different milk vending machines supplied by different milk producers located in Zagreb, Croatia (from the Savica (45.79498787749926 N 15.995354074642366 E), Dugave (45.765381911857425 N 15.99328086175341 E), and Jelkovec (45.81056119505793 N 16.10324176615681 E) markets) and Velika Gorica, Zagreb County (one in front of the school (45.719049726057904 N 16.074359705074272 E) and another in front of the health center (45.71389808810045 N 16.0655078885702 E)). Raw milk was sampled seven times (in duplicate) for each milk vending machine over 3 months (February, March, and April 2023) [25]. The milk was stored in 1 L sterile bottles with lids and submitted for analysis no later than one hour after sampling. The temperature of milk during transport was +6 °C (±2 °C).

### *2.2. Flow Cytometry Method and Plate Count Method*

The total number of bacteria (CFU/mL) in raw milk samples was determined by the flow cytometry method on the Bactoscan FC type 73700 instrument (Foss, Hillerød, Denmark) according to the international norm ISO 21187:2021 [26] in the Reference Laboratory for Milk and Dairy Products, Department of Dairy Science, Faculty of Agriculture, University of Zagreb. This was performed to facilitate the selection of the appropriate dilution for the standard method according to the international norm ISO 4833-1:2013 [24] for determining the number of aerobic mesophilic bacteria. Both mentioned methods are accredited in accordance with the requirements of the international standard ISO 17025:2017 [27].

### *2.3. MALDI-TOF Mass Spectrometry*

A sample of the grown bacterial colony was taken from the Petri dish using a sterile toothpick and applied to the MALDI plate. Each sample was overlaid with 1 µL of 70% formic acid (Fisher Chemical, Alcobendas, Spain) and dried at room temperature. Then, 1 µL of the MALDI matrix (10 mg/mL alpha-4-cyano-4-hydroxycinnamic acid; Bruker Daltonik, Bremen, Germany) was added to the sample, and the sample was dried again.

Microorganism identification was performed using a Microflex LT mass spectrometer (Bruker Daltonik, Bremen, Germany) and the MBT Compass HT version 5.1 (Bruker Daltonik, Bremen, Germany) software by comparing the recorded MALDI-TOF spectra of the sample with a reference spectrum stored in the database version 11. According to the manufacturer's instructions, the identification criteria were as follows: values from 2.00 to 3.00 indicate high confidence identification, values from 1.70 to 1.99, low confidence identification, and for values below 1.69, identification is not reliable [28].

### *2.4. Data Analyses*

Analysis of variance (ANOVA) was performed and the Mann–Whitney U test was used (significance level 0.05) to determine the differences among mean values of raw drinking milk from different vending machines, during two seasons.

As a multivariate tool for the analyses of qualitative data, the heatmap was used with the color scale ranging from white (not identified) to yellow (low certainty of identification) to green (high certainty of identification). The quantitative data were additionally also explored by the use of multivariate tools, and principal component analysis (PCA) was used. This analysis enabled insight into which raw drinking milk samples (from different vending machines) had the highest incidence of bacteria (Gram-positive, Gram-negative), yeasts, and molds, and whether the occurrence of bacteria can be related to the seasons (four measurements during winter and three measurements in the spring months). Classification trees were used with the aim of associating the occurrence of certain species of bacteria, yeasts or molds in milk samples from vending machines, depending on whether the CFU is within the recommended limits (<100,000 CFU/mL) or not. The statistical software tool for Excel (XLStat, 2007) was used for all qualitative and quantitative analyses presented in this study.

## 3. Results and Discussion

In this study, the hygienic quality of raw drinking milk sold through five milk vending machines in the City of Zagreb and Zagreb County was analyzed using the flow cytometry method to determine the total number of bacteria and the classical method to determine aerobic mesophilic bacteria (Table 1). The MALDI-TOF MS technique was used to identify microbial populations (Tables 2 and 3).

**Table 1.** The total number of bacteria determined in milk from different vending machines during the winter and spring months, (mean values with corresponding standard errors) by BactoScan.

| Milk Vending Machines | CFU × $10^3$/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| | 6 February 2013 | 13 February 2023 | 20 February 2023 | 27 February 2023 | 6 March 2023 | 27 March 2023 | 3 April 2023 |
| | W1 | W2 | W3 | W4 | S1 | S2 | S3 |
| 1st | $53 \pm 4^{A,b}$ | $969 \pm 125^{A,d}$ | $82 \pm 1^{A,b,c}$ | $292 \pm 2^{C,c}$ | $7 \pm 4^{A,a}$ | $54 \pm 2^{B,b}$ | $170 \pm 1^{A,c}$ |
| 2nd | $387 \pm 16^{C,b}$ | $446 \pm 55^{A,b}$ | $84 \pm 4^{A,a}$ | $112 \pm 6^{A,a}$ | $5270 \pm 562^{D,d}$ | $457 \pm 1^{D,b}$ | $3818 \pm 39^{C,c}$ |
| 3rd | $169 \pm 3^{B,b}$ | $12 \pm 1^{B,a}$ | $248 \pm 2^{B,c}$ | $98 \pm 1^{A,a,b}$ | $82 \pm 6^{B,a,b}$ | $188 \pm 5^{C,b}$ | $307 \pm 13^{B,c}$ |
| 4th | $48 \pm 2^{A,b}$ | $15 \pm 0^{B,a}$ | $685 \pm 8^{C,d}$ | $208 \pm 1^{B,c}$ | $242 \pm 44^{C,c}$ | $26 \pm 2^{A,a}$ | $246 \pm 21^{B,c}$ |
| 5th | $60 \pm 2^{A,a}$ | $15 \pm 2^{B,a}$ | $305 \pm 20^{B,b}$ | $196 \pm 2^{B,b}$ | $5830 \pm 0^{D,d}$ | $1753 \pm 72^{E,c}$ | $167 \pm 2^{A,b}$ |

Different capital letters in the columns indicate significant differences in the number of bacteria detected in different vending machines, on the same date; different small letters in the rows indicate significant differences for bacterial numbers on different dates, per vending machine. Significance level, $p < 0.05$.

In the presentation of the total number of bacteria (Table 1), instead of standard deviation, standard error (SE) was used because this parameter provides information on data reliability because it presents the ratio of standard deviation to the root of the number of measurements. As can be seen from the results, the SE value in season S1 is extremely high for the second VM (562 CFU × $10^3$/mL) based on the total number of bacteria, which ranges from 471 to 583 CFU × $10^3$/mL. According to data from the Agricultural and Food Agency of the Republic of Croatia (HAPIH) in 2023 (Center for Quality Control of Livestock Products, Annual Report 2024) [29], 97.1% of milk belongs to the first category, which is why the requirement of a total microbial count of ≤100,000 CFU/mL is included in the framework of the control of the hygienic quality of milk and is also used for the classification and determination of milk prices. When analyzing the results of bacterial counts from individual vending machines, a very wide range of populations was present, with levels above the legal limits. However, this is not a surprising finding due to factors such as

sampling time point (the period from milking to sampling) or hygienic and temperature conditions during milk collecting, storage, and transport. The same differences were noted in our previous study [13]. Unfortunately, the exact duration of the period from milking to sampling was not known in this study. If we assume that all samples were equally fresh at the time of sampling, the observed variability in bacterial counts between the machines could be due to poor hygiene or udder health problems. On the other hand, a higher TVC value does not necessarily mean that pathogens are present, and vice versa.

The total viable count itself does not have any significance in evaluating the safety of milk. However, many foodborne pathogens and spoilage microorganisms are capable of growing in non-selective nutrient mediums and under the incubation conditions applied for TVC determination (ISO 4833-1:2013). Therefore, this study was conducted to analyze the types of microbes present in raw milk collected from different vending machines using the MALDI-TOF mass spectrometer. A total of 700 selected colonies based on different morphotypes were analyzed. The use of the MALDI-TOF method in microbial identification is therefore culture-dependent, i.e., it relies on the prior isolation of viable cells, in this case, on nutrient plate count agar. Another factor in assessing the composition of the microbial population is the strategy of sampling microbial colonies as representatives of the population, which is preferably performed randomly. In this sense, the probability of identifying pathogens within the TVC population depends on these two factors. The results of microbial identifications of Gram-negative bacteria are listed in Table 2, Gram-positive bacteria in Table 3, and sporadic yeasts and molds in Table 4.

A very high species diversity of Gram-negative bacteria was found in raw milk from vending machines. In general, the natural microbiota of milk consists mainly of Gram-positive species, so the finding of the listed enterobacteria and other Gram-negative species indicates environmental or fecal contamination. The predominant species was the enterobacterium *Hafnia alvei*, followed by coliform *E. coli* and psychrophilic *Rahnella inusitata*. Remarkably, *E. coli* and *H. alvei* were found in most samples, but almost every sample from vending machine (VM) 3 contained *E. coli*, indicating poor hygienic practice. The presence of *E. coli* in raw milk has some indirect significance for food safety if the population consists of pathogenic strains or serotypes [5]. A recent study [30] has confirmed the public health significance of *E. coli* in raw milk due to the occurrence of virulent and multi-drug resistant strains. In addition, *Yersinia enterocolitica* has been identified sporadically in vending machines, indicating a potential risk as it is a psychrophilic foodborne pathogen that has been implicated in foodborne outbreaks traced to milk, including pasteurized milk, in the past [31]. *Pseudomonas* species were frequently identified in VM milk, indicating their possible growth during milk refrigeration, as they are the main psychotropic and spoilage bacteria in raw milk. This can be related to the results of our previous study conducted on farm milk samples [32], in which the presence of pseudomonads was rarely detected by MALDI-TOF MS. The detection of contaminating opportunistic pathogens from the Sphingobacteriaceae family and *Serratia* genus, which have also been identified in milk by others [32–35], albeit only sporadically, should be emphasized.

Gram-positive bacteria were dominated by the species *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Carnobacterium maltaromaticum*, which are frequently found in raw milk and have already been reported by Quigley et al. [36], Mikulec et al. [32], and others. Lactic acid bacteria are generally recognized as safe (GRAS) and are beneficial microbes (probiotic properties). However, their acidifying capacity in chilled raw milk can lead to spoilage of the milk if they are present in large numbers. Their numbers usually increase from milking to consumption, even if the cold chain is not interrupted [13]. In addition, staphylococci and streptococci, which are of public health importance, were detected in 10–15% of the samples. For example, *Staphylococcus aureus*, as one of the most common pathogens causing

mastitis in cows, but also pathogenic in humans, and *S. haemolyticus*, as an opportunistic pathogen in animals and humans [37]. The public health significance of *S. aureus* in milk and cheese produced from unpasteurized milk has been widely reported, including the multi-resistant strains as well enterotoxin produced in highly contaminated products [38].

In addition, our study also identified animal pathogens (mastitis pathogens) in vending machine milk, such as *Streptococcus uberis*, *S. parauberis*, and *Streptococcus dysgalactiae*, indicating possible udder health disorders in a few dairy farms [39–41].

**Table 2.** Gram-negative bacteria detected in raw milk samples from five vending machines during winter (W1–W4) and spring (S1–S3) time.

| Gram-Negative Bacteria | W1 | | | | | W2 | | | | | W3 | | | | | W4 | | | | | S1 | | | | | S2 | | | | | S3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| *Acinetobacter albensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter guillouiae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter johnsonii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Acinetobacter parvus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Aeromonas bestiarum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Aeromonas eucrenophila* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Aeromonas salmonicida* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Brevundimonas diminuta* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Buttiauxella gaviniae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Buttiauxella warmboldiae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium bovis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium indoltheticum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium piscium* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium scophthalmum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium shigense* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Chryseobacterium vrystaatense* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Citrobacter braakii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Comamonas terrigena* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Escherichia coli* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Hafnia alvei* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Janthinobacterium lividum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Klebsiella oxytoca* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus curvatus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Moraxella osloensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pantoea agglomerans* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Paracoccus yeei* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas azotoformans* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas brenneri* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas extremorientalis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas fluorescens* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas gessardii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas libanensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas lundensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas proteolytica* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas rhodesiae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas synxantha* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pseudomonas tolaasii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

**Table 2.** *Cont.*

| Gram-Negative Bacteria | W1 | | | | | W2 | | | | | W3 | | | | | W4 | | | | | S1 | | | | | S2 | | | | | S3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| *Pseudomonas veronii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Rahnella inusitata* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Serratia marcescens* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Serratia proteamaculans* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Sphingobacterium faecium* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Sphingobacterium multivorum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Stenotrophomonas maltophilia* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Stenotrophomonas spp.* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Yersinia enterocolitica* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Yersinia enterolitica* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Green fields: high reliability of identification (in the range ≥ 2.00 to 3.00); yellow fields: low reliability of identification (range of 1.70 to 1.99); white fields: not detected.

**Table 3.** Gram-positive bacteria detected in raw milk samples from five milk machines during winter and spring time.

| Gram-Positive Bacteria | W1 | | | | | W2 | | | | | W3 | | | | | W4 | | | | | S1 | | | | | S2 | | | | | S3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| *Bacillus licheniformis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Brochothrix thermosphacta* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Carnobacterium maltaromaticum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Corynebacterium callunae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Corynebacterium frankenforstense* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Corynebacterium provencense* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Corynebacterium vitaeruminis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Corynebacterium xerosis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Enterococcus faecalis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Enterococcus hirae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Kocuria uropygioeca* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Kocuria varians* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lacticaseibacillus paracasei* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus curvatus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lactobacillus spp* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lactococcus lactis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

**Table 3.** *Cont.*

| Gram-Positive Bacteria | W1 | | | | | W2 | | | | | W3 | | | | | W4 | | | | | S1 | | | | | S2 | | | | | S3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| *Lactococcus plantarum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Lactococcus raffinolactis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Leuconostoc carnosum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Leuconostoc citreum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Leuconostoc mesenteroides* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Luteococcus japonicus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Microbacterium aurum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Microbacterium lacticum* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Microbacterium liquefaciens* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Microbacterium oxydans* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Micrococcus luteus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Rhodococcus baikonurensis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus aureus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus borealis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus chromogenes* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus haemolyticus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus vitulinus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Staphylococcus warneri* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Streptococcus dysgalactiae* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Streptococcus parauberis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Streptococcus uberis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Streptomyces albidoflavus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Green fields: high reliability of identification (in the range ≥ 2.00 to 3.00); yellow fields: low reliability of identification (range of 1.70 to 1.99); white fields: not detected.

Yeasts and molds were also detected in the raw milk samples. They are naturally present in raw milk and can contribute to the spoilage of milk during cold storage on the market. They were randomly selected from the nutrient agar (they grow under aerobic conditions), but the colonies were significantly larger compared to the bacterial colonies. The effectiveness of the MALDI-TOF MS method in their detection and identification within the milk microbiota is an added value in microbial screening [13,32].

**Table 4.** Yeasts and molds detected in raw milk samples from five milk machines during winter and spring time.

| Yeasts and Molds | W1 | | | | | W2 | | | | | W3 | | | | | W4 | | | | | S1 | | | | | S2 | | | | | S3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th | 1st | 2nd | 3rd | 4th | 5th |
| *Scopulariopsis brevicaulis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Kluyveromyces lactis* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Kluyveromyces marxianus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Magnusiomyces capitatus* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pichia fermentans* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Pichia kudriavzevii* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| *Yarrowia lipolytica* | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Green fields: high reliability of identification (in the range $\geq$ 2.00 to 3.00); yellow fields: low reliability of identification (range of 1.70 to 1.99); white fields: not detected.

Heat maps provided a clear qualitative insight into the seasonal (different) occurrence of bacteria, yeasts, and/or molds. However, in order to clarify the significance of the relationship between seasonality and raw drinking milk quality from different vending machines, it was necessary to apply some multivariate analysis tools.

Principal component analysis (PCA) is a widely used multivariate statistical technique in various fields, including food science and technology [42] and became a widely used method in food quality monitoring [43]. This multivariate tool is used to reduce the dimensionality of complex data, such as chemical, physical or sensory food analyses, allowing researchers to identify the main factors that affect the food quality [44]. The application of this method is growing thanks to the constantly expanding availability of software that enable complex analyses and whose qualitative and quantitative results can be used in monitoring food quality, especially in the context of ensuring food safety and meeting regulatory standards [42–44]. Milk, as a complex and heterogeneous food matrix, has been a subject of interest in the application of principal component analysis within the domain of food quality. The complex nature of milk, with its numerous quality parameters, creates challenges in data interpretation and reduction, and PCA analysis can be a valuable tool in addressing these challenges, as it allows for the identification of the most important variables that contribute to the overall milk quality and the reduction in the dimensionality of the dataset [45]. In the context of dairy farming, PCA has proven useful for analyzing and optimizing the quality of dairy products [46] and in the identification of key factors that affect the composition, texture, taste, and nutritional value of milk and dairy products [47]. The method is also important in detecting contamination [48], distinguishing products by origin [49] or production technology [50], and assessing authenticity [51]. In addition to seasonal variations, principal component analysis has also been applied to investigate the relationship between various physicochemical and microbiological properties of milk [32], and to develop models for predicting milk quality characteristics [52]. Following all of the previously mentioned considerations, we used PCA analysis to determine the connection between seasonality and milk quality, if CFU is observed.

From the tables, the trend of occurrence at the same vending machines is visible; therefore, a multivariate analysis was applied to determine the qualitative trend towards different vending machines and whether this is also related to the seasonal character (when cows are potentially grazing, etc.). This is indicated in Figure 1A,B.

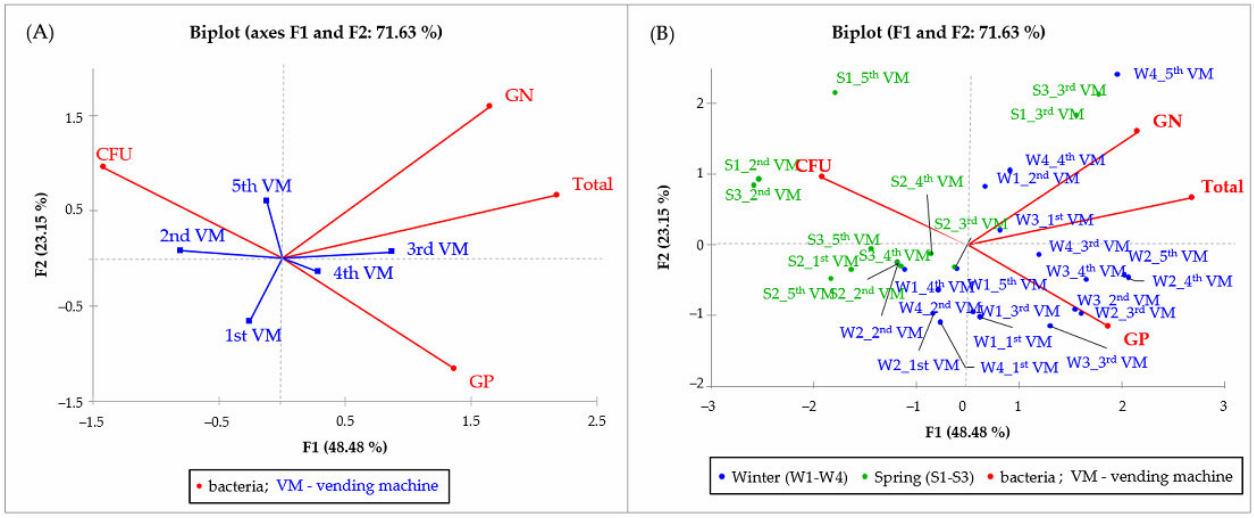


**Figure 1.** Principal component analysis of microorganism detection and bacterial count (total, Gram-negative (GN), and Gram-positive (GP)) across five vending machines (VM1–VM5) (**A**) during different seasons (winter: W1–W4 and spring: S1–S3) (**B**).

Only two principal components (F1 and F2) describe a high share (71.63%) of the variations in the observed data set. The PCA investigation of the identified number of certain species, total bacterial number, and the CFU per mL in the raw drinking milk samples from different vending machines showed that all observed parameters are distributed in all four quadrants. Thus, according to Figure 1A, the lowest frequency of detection of Gram-negative bacteria was for the raw milk samples from the first vending machine, which are positioned in the third quadrant, the opposite quadrant to the one in which the variable GN (number of Gram-negative bacteria) was positioned. Figure 1B shows that season is also a key parameter because the majority of the green marks (spring season, S1–S3) clustered together, as did the blue marks (winter season, W1–W4). This qualitative insight prompted us to further analyze the number of species and CFU/mL for each raw drinking milk sample from five vending machines according to the measurement dates (seasonal character) (Figure 2).

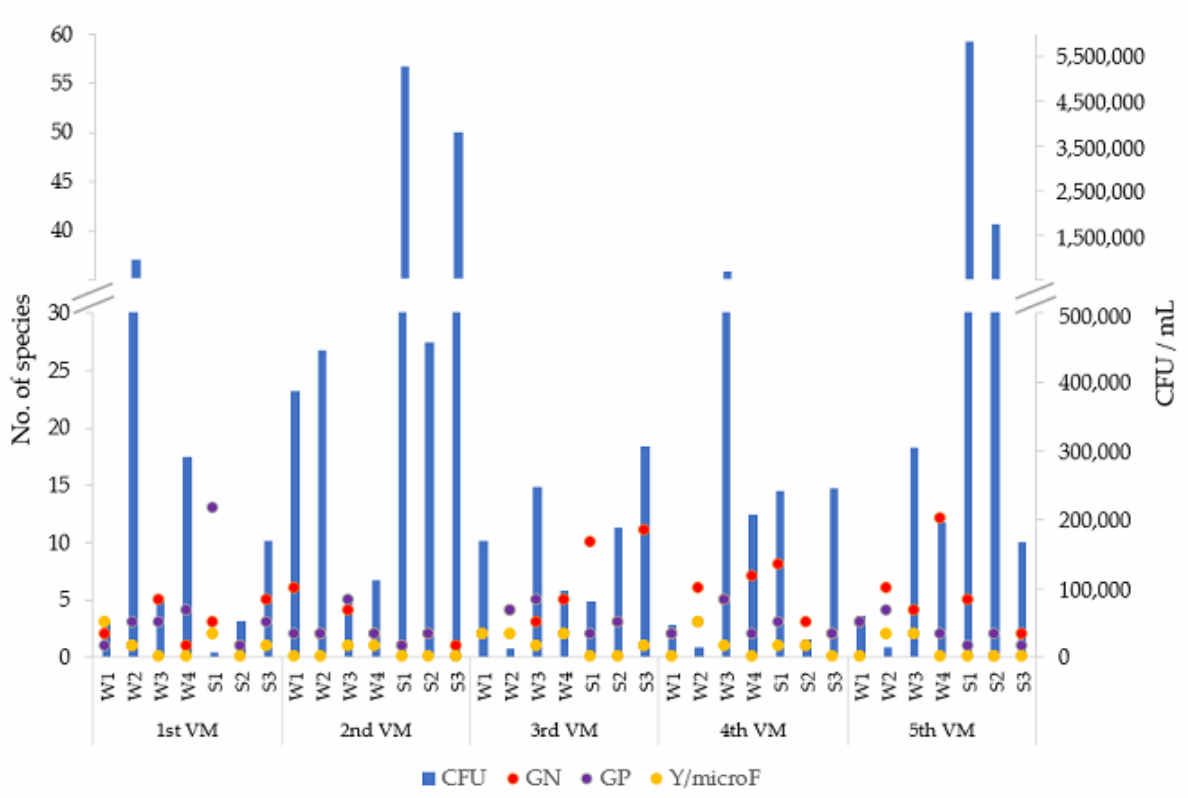


**Figure 2.** Variation in detected microorganism and total bacterial count across raw drinking milk from five vending machines (VM1–VM5) during two seasons (winter: W1–W4 and spring: S1–S3).

As our data set included qualitative and quantitative data, as presented in previous figures and tables, all the data processing methods used were subject to this. Thus, the heat map and PCA enabled a qualitative insight into the different representations of bacterial genera in different seasons. However, the issue we particularly wanted to address is the relationship between the number of bacteria (CFU < 100,000) and the expected species of bacteria, because it is by no means desirable that bacteria, yeasts, and/or molds that can endanger human health appear at low CFU, which would be considered as highly acceptable. Therefore, the Clustering tree method was used. This method is also known as the hierarchical grouping method and it is used for the organization and analysis of large data sets (such as the species of bacteria, yeasts, and molds in this investigation) by creating tree structures that visualize relationships between observed groups. In this method, the grouping of data according to similarities and differences is observed along

with a quantitative assessment of expectations. The method was chosen because Clustering tree is widely used in the food sector. It is used to classify products according to sensory, chemical, and nutritional characteristics [53]. In dairy products, it allows the separation of different types of milk based on parameters such as microbiological availability [54]. Also, this method helps in the optimization of production processes by grouping performance data, identifying key variables, and facilitating the adaptation of recipes or processing techniques [55]. Visually, the tree representation facilitates the interpretation of complex relationships between data, which is particularly useful for strategic decision-making because it indicates the factors that most influence the observed process. Unlike classical clustering methods, Clustering tree offers flexibility because it allows data analysis at different levels hierarchies. This allows users to examine relationships between groups in detail at a high level or focus on specific subgroups.

Thus, in Figure 3, by the use of Clustering tree, the occurrence of bacteria species if the CFU is in the range of the recommended value (<100,000 CFU per mL) is presented. There is an 18% probability that the value of total bacteria will be less than 100,000 CFU/mL and that bacteria from the genus *Staphylococcus* will be present, while the probability for *Yarrowia* spp. and *Chryseobacterium* spp. is 6%. A table indicating the probability has been added to the graphic display occurrence of a certain strain of bacteria depending on whether the total number of bacteria is in accordance with the recommendations ("Yes", orange mark) or not ("No", blue mark). The Clustering tree shows that *Yarrowia* spp. and *Chryseobacterium* spp. will appear in 50% of raw milk samples if *Staphylococcus* spp. is not detected.

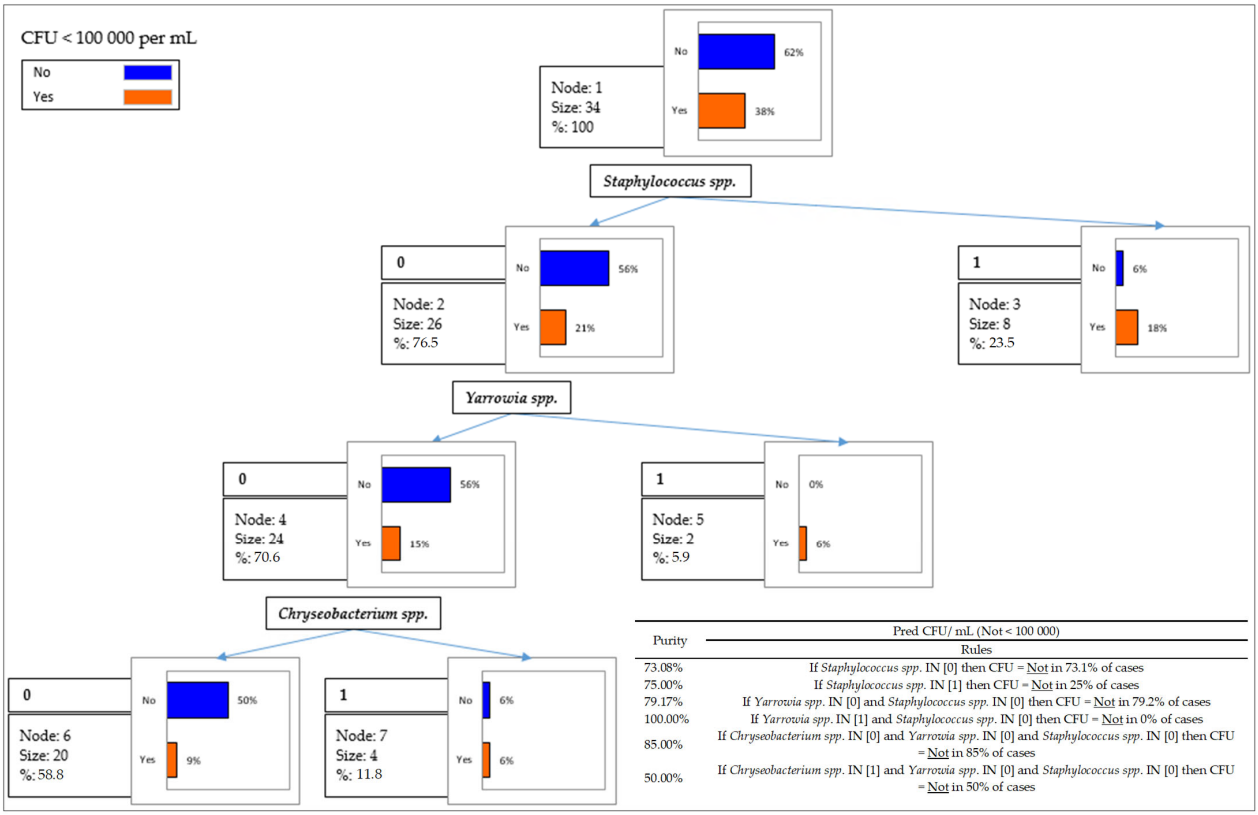


**Figure 3.** Clustering tree with prediction of expectations of individual strains depending on whether CFU < 100,000 per mL.

Our study followed the sequence of qualitative data analysis (heat map), combining qualitative and quantitative data (PCA and Clustering tree) in data processing. The results of this research confirm the advantages of applying multivariate tools in the analysis of

qualitative and quantitative data, because it is precisely the integration of methods such as heat maps, PCA analysis, and Clustering tree that improves the precision and efficiency of the information derived from the results of processing such data [56,57].

All the tools used in data processing have shown that they are powerful tools that in any sector, as well as in the food industry, product quality monitoring, etc., enable better understanding and management of complex product quality data, which contributes to new knowledge. The Clustering tree provided us with an extremely useful insight into the occurrence of bacteria independent of the total CFU, which is extremely important in ensuring food safety and compliance with regulatory standards.

## 4. Conclusions

In conclusion, this research identifies a diverse microbiota in milk from vending machines by MALDI-TOF MS, including 38 bacterial genera and five fungal genera, with prominent species such as *Lactococcus lactis*, *Hafnia alvei*, *Escherichia coli*, and *Leuconostoc mesenteroides*. In addition to *E. coli*, the microbial population consisted of foodborne pathogens such as *S. aureus* or *Y. enterocolitica*, indicating potential risk from raw milk consumption. By using a Clustering tree analysis on the MALDI-TOF MS data, it was shown that some pathogens may occur in raw milk even if the total viable count is low. This emphasizes the need for stricter control of raw milk from vending machines by obligatory testing of the presence of specific foodborne pathogens. Even the nutritional value of raw milk is not questionable; our results imply the potential health risk for consumers due to the presence of pathogens and the obligation of thermal processing before consumption.

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