Correlation between Microbial Population and Oxidative Stability of the Yogurt-Based Tzatziki Salad

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Abstract: Tzatziki is a deli salad widely consumed in the Eastern Mediterranean and Balkan countries, and it is acknowledged for its health benefits. So far, it is proved to exhibit extreme resistance to microbial spoilage by (pathogenic) microorganisms and microbial self-stabilization, but no research was carried out regarding its oxidative stability despite the fact that it contains a large amount of lipids. In this study, the factor that affects the oxidative stability of tzatziki was exploited. Different samples of tzatziki salad were prepared and stored for 16 or 27 days, depending on the conducted experiment. They varied in the type of yogurt (set yogurt or traditional Greek-style yogurt), the type of oil (olive oil or soybean oil), and the addition or not of preservatives, garlic, and cucumber. Samples were analyzed in terms of oxidative stability (by the Rancimat method), colony-forming units, and tocopherol content throughout the storage period. Among the examined parameters, no correlation between the tocopherol content and oxidative stability was recorded. However, a strong correlation between the microbial population and the oxidative stability was recorded. Therefore, this correlation can be used to prepare tzatziki salads with increased shelf life and decreased flavor deterioration (due to oxidation). Moreover, such correlations should be further exploited for other foods so as to promote their stability.

Keywords: antioxidant activity; deli salad; lactic acid bacteria; microorganisms; oxidation; Rancimat; shelf life; tocopherols

1. Introduction

Tzatziki is a deli salad widely consumed in East Mediterranean and the Balkan countries [1–5]. It is a mixture of yogurt (usually from sheep or goat milk), vegetable oil, freshly cut cucumber, garlic, salt, and dill [6,7]. It is generally acknowledged for its health properties deriving mainly from the garlic and cucumber, which exhibit antibacterial, antiviral, and antifungal activity, besides their benefits on the cardiovascular and immune system [2,8,9]. In addition, due to its taste, tzatziki has been traditionally consumed for more than a hundred years as an appetizing dish or accompanying meals rich in fat.

Tzatziki is usually stored aerobically under refrigeration (4 °C) and has an estimated shelf life of 3–4 weeks. Although it has a high moisture content (due to its ingredients: yogurt and cucumber) and low salt content, it was proved that it not only exhibits extreme resistance to microbial spoilage, but it also microbiologically self-stabilizes after a short period [1]. It is noteworthy that despite the lipid content of tzatziki, no oxidation odors are reported by consumers. Oxidation of lipid ingredients in foods causes the formation of hydroperoxides, which in turn are not only more susceptible to oxidation, but also lead to the formation of decomposition products such as aldehydes and ketones, which bestow a negative aroma and taste to the products. This has a negative impact both on the nutritional...
value of the product, as well as on the acceptance by consumers. As such, industries try to create oxidative-stable products that have extended shelf life [10–12]. In this context, various methods have been employed to avoid lipid oxidation, including addition of antioxidant compounds such as tocopherols or plant extracts, the use of modified atmosphere or vacuum to package the food product, the use of edible films and coating [13,14], etc.

In this study, we examined the stability of tzatziki salad against oxidation. To the best of our knowledge, there are no previous studies that have examined the origin of tzatziki’s stability against oxidation. In an effort to shed light on this, tzatziki salads prepared using various ingredients were examined. Moreover, the tocopherol content of the salad (a common antioxidant compound with exceptional antioxidant properties for lipids) as well as the microbial population were examined.

2. Materials and Methods

2.1. Food Ingredients

Tzatziki samples (S1–S4) and tzatziki model systems (M1 and M2) were prepared at the laboratory using various recipes with or without preservatives (sodium benzoate and potassium sorbate). Set sheep yogurt (fat content 6.4%), traditional Greek-style sheep yogurt (fat content 6.6%), cucumber, garlic, vinegar, soybean, virgin olive oil, salt, wheat starch, guar, and xanthan gum used for the various recipes were all purchased from a local market in Karditsa, Greece.

2.2. Reagents

The reagents (analytical and HPLC grade) used for the extraction of lipid phase and the determination of tocopherols were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions of tocopherols were purchased from Merck Ltd. (Darmstadt, Germany). The reagents used for the determination of total microbial count (Ringer solution and Total Plate Count agar) were also obtained from Merck Ltd.

2.3. Instruments

A Bosch MC812M865 (Robert Bosch GmbH, Stuttgart, Germany) food processor was used for the preparation of the samples. A stomacher (Seward, VWR, Edmonton, AB, Canada) was used for homogenization of the samples. For calculation of the induction periods, a Rancimat 679 (Metrhom LTD, Herisau, Switzerland) was employed. HPLC analysis of the samples was carried out on a Shimadzu Prominence CBM-20A liquid chromatograph (Shimadzu Europa GmbH, Duisburg, Germany) equipped with SIL-20AC autosampler and a CTO-20AC column oven. Detection was performed using a Shimadzu RF-10AXL fluorescence detector set at 278 nm (excitation) and 339 nm (emission). The mobile phase consisted of n-hexane:2-propanol:absolute ethanol (97.5:2:0.5, v/v/v) and the flow rate was set at 1.0 mL per min. The column used was a Waters µ-Porasil (125 Å, 10 µm, 3.9 × 300 mm) (Waters Corp., Waltham, MA, USA). Chromatographic separation was carried out at 30 °C.

2.4. Recipes

Four different tzatziki samples (S1, S2, S3, and S4) were prepared following a traditional recipe using the proportions presented in Table 1. The oil (soybean or virgin olive oil) was poured into the yogurt (set or traditional Greek-style) and mixed in a food processor until well combined. Then, cucumber and garlic were peeled and cut into pieces (no longer than 1 cm). Finally, cucumber, garlic pieces, and vinegar were mixed with oil and yogurt for 5 min. The mixtures were preserved at 4 °C for 27 days. Additionally, two tzatziki model systems (M1 and M2) were prepared and preserved at 4 °C for 16 days. In the first case, samples were stored for 27 days, aiming for a longer storage period, so as to examine the oxidation degree, while in the second case, samples were stored for 16 days, aiming for the examination of CFU and oxidation degree. The proportions used are presented in Table 2.
Table 1. Proportions used for the preparation of samples S1, S2, S3, and S4.

<table>
<thead>
<tr>
<th>Tzatziki Sample</th>
<th>Soybean Oil (% w/w)</th>
<th>Virgin Olive Oil (% w/w)</th>
<th>Set Sheep Yogurt (% w/w)</th>
<th>Traditional Greek-Style Sheep Yogurt (% w/w)</th>
<th>Vinegar (% w/w)</th>
<th>Cucumber (% w/w)</th>
<th>Garlic (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>9</td>
<td>-</td>
<td>67</td>
<td>5</td>
<td>17</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>67</td>
<td>5</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>S3</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>67</td>
<td>5</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>S4</td>
<td>-</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>5</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Proportions used for the preparation of samples M1 and M2.

| Ingredient |  | M1 (% w/w) | M2 (% w/w) |
|------------| |------------|------------|
| Set sheep yogurt | | 43.5       | 43.5       |
| Cucumber   | | 24.8       | -          |
| Water      | | 17.1       | 43.2       |
| Soybean oil| | 8.4        | 8.4        |
| Wheat starch| | 2.9        | 2.9        |
| Salt       | | 1.0        | 1.0        |
| Vinegar    | | 0.9        | 0.9        |
| Garlic     | | 1.2        | -          |
| Preservatives (sodium benzoate/potassium sorbate) | | 0.05/0.05 | -          |
| Guar gum   | | 0.05       | 0.05       |
| Xanthan gum| | 0.05       | 0.05       |

2.5. Determination of Colony-Forming Units (CFUs)

The determination of CFU/g was carried out as described in previous work [1]. In brief, 25 g of tzatziki sample was mixed with 225 mL of ringer solution and homogenized with a stomacher. After incubation for 1 h at 25 °C, tenfold dilutions were prepared, and an appropriate amount of the last dilution was plated on Total Plate Count agar plates. After incubation at 37 °C, the number of colonies was measured, and results are expressed as average (at least five plates were counted for each sample) CFUs per g of sample. For samples S1–S4, CFUs were determined on days 1, 3, 6, 8, 10, 14, 20, 23, and 27. For samples M1 and M2, CFUs were determined on days 1, 6, 10, 13, and 16.

2.6. Extraction of Fat

The extraction of the lipid phase from tzatziki preparations was carried out according to the method described by Official Methods of Analysis of AOAC, No. 935.60 [15].

2.7. Determination of Oxidation Stability

The susceptibility to oxidation of the extracted fat was determined using a Rancimat 679. The temperature was set at 90 °C and the airflow at 15 L/h. Results are expressed as induction period (hours).

2.8. Determination of the Tocopherol Composition

The tocopherol (vitamin E) content of the lipid phase of tzatziki samples was determined as previously described [16]. In brief, 1 g of the lipid phase was transferred to an amber glass vial and 5 mL of n-hexane was added. After vortexing for 1 min, the sample was filtered via a PVDF syringe filter and 20 μL of the sample was injected to the HPLC system.

2.9. Statistical Analysis

All recipes where prepared in three independent replicates (using different batches of ingredients each time) and each assay was also carried out in triplicate. Results are expressed as the average of nine measurements. Statistical significance of the differences
between mean values was assessed by ANOVA, followed by Duncan’s multiple range test, for \( p < 0.05 \). Pearson correlation analysis between the CFU and the induction period was also carried out. Statistical analysis was carried out using IBM SPSS Statistics (version 26) (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

As shown by our previous work, tzatziki, a traditional food of the East Mediterranean region, proved to be resistant to microbial spoilage [1]. In addition, despite its oil content reaching nearly 10%, no oxidation odors were reported, while in similar emulsified foods, oxidative damage of the lipid phase occurs in a very short time, decreasing the nutritive and organoleptic quality of the final product. Along with oxidative rancidity, hydrolytic rancidity may also occur in tzatziki salad. However, if hydrolytic rancidity took place, off-flavor compounds would also be produced [17]. Considering that the oxidation products (either from hydrolytic or oxidative deterioration) are highly detectable due to their extreme odor, the unpleasant flavor is easily detectable even if the fat phase content is relatively low. However, this is not typical for tzatziki salad, nor did it occur in our case. This could be attributed to the addition of garlic and cucumber that contain natural antioxidant compounds that either hinder or lower the rate of rancidity progress. Moreover, hydrolytic rancidity would result in increase in the pH of tzatziki salad. This would result in increased syneresis of the yogurt in the tzatziki salad, which was not apparent in our case. To further examine if this is the case, the pH of tzatziki samples within 16 days of storage was recorded (tzatziki salad was diluted with distilled water (1:1 \( w/v \)), agitated for 30 min, centrifuged, and pH was recorded). We found that it decreases from 4.3 to 4.1, which is not indicative of hydrolytic rancidity, since a similar decrease in the pH can be observed in yogurts or tzatziki salad [18].

Thus, in order to examine whether the oil phase of tzatziki is oxidative-stable, and if this oxidative stability is attributed to the ingredients used, different ingredients were examined for samples S1–S4. The induction periods (IP) of the samples S1–S4 along with those of the oils used in the recipes (virgin olive oil and soybean oil), obtained by the Rancimat method, are presented in Table 3. As can be seen, all tzatziki samples exhibited increased IP (statistically significant for \( p < 0.05 \)) compared to the respective control samples (soybean oil was used for samples S1 and S2 and virgin olive oil was used for samples S3 and S4). It is also evident that samples prepared with virgin olive oil (S3 and S4) were the most resistant to oxidation (significant at \( p < 0.05 \)). Among samples S3 and S4, the preparation with the set sheep yogurt (S3) showed the least oxidation (significant at \( p < 0.05 \)). Similar were the results regarding the lipid phases extracted from the tzatziki samples made with soybean oil (S1 and S2). The preparation with the set sheep yogurt (S1) showed greater resistance (significant at \( p < 0.05 \)). Therefore, two parameters were found to affect the oxidative stability of tzatziki salad: the type of oil and the type of yogurt used. As regards to the type of oil, examination of the respective oil samples showed that the virgin olive oil has increased IP compared to soybean oil, and as such, its use was expected to result in tzatziki samples with increased oxidative stability. As regards to the type of yogurt used, both yogurts were made from sheep milk, albeit prepared using a different method. From previous studies, it was shown that these two types of yogurts have different content in lactic acid bacteria (LAB), which in turn can result in differences in the composition, such as the content in organic acids (e.g., lactic acid, citric acid, etc.), lactose, and other organic compounds [19–22]. This was also found in our case (vide infra), suggesting a relation between the content of LAB and oxidative stability. In addition to the above, the tocopherol content of all samples was examined. As can be seen, all tzatziki samples contain fewer tocopherols from the respective oils used for the preparation. This was anticipated, since other ingredients are also added to prepare tzatziki salad, resulting either in side reactions with compounds from the other ingredients, or in consumption in oxidative reactions. However, no correlation was found between the IP and the content of \( \alpha-, \gamma-, \) and \( \delta- \)tocopherol, or the total tocopherol content.
Table 3. Rancimat induction period and tocopherol content of the lipid phase extracted from tzatziki samples after 27 days of preservation at 4 °C. Statistically significant differences (for $p < 0.05$) between sample and control are denoted with *.

<table>
<thead>
<tr>
<th>Lipid Phase of Samples</th>
<th>Induction Period (h)</th>
<th>Tocopherol Content (mg/kg of Lipid Phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\alpha$-Tocopherol</td>
</tr>
<tr>
<td>Soybean oil (control)</td>
<td>33.0 ± 0.8</td>
<td>239.1 ± 11.3</td>
</tr>
<tr>
<td>S1</td>
<td>45.9 * ± 1.1</td>
<td>204.2 ± 12.6</td>
</tr>
<tr>
<td>S2</td>
<td>42.1 * ± 1.1</td>
<td>178.7 * ± 11.9</td>
</tr>
<tr>
<td>Virgin olive oil</td>
<td>40.0 ± 1.0</td>
<td>160.4 ± 10.7</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td>126.7 * ± 9.8</td>
</tr>
<tr>
<td>S3</td>
<td>62.8 * ± 1.4</td>
<td>93.1 * ± 9.0</td>
</tr>
<tr>
<td>S4</td>
<td>54.1 * ± 1.3</td>
<td>126.7 * ± 9.8</td>
</tr>
</tbody>
</table>

In an effort to shed light on the parameter that affects the oxidative stability of tzatziki samples, the microbial population of the samples was assessed on different days, along with the IP. The results are presented in Figure 1. It can be seen that samples prepared with set sheep yogurt contained more CFU/g compared to the microbial population of samples prepared with traditional Greek-style sheep yogurt. As indicated by the results, as the amount of CFU/g of microorganisms in the samples was decreasing with storage time, the IP was also found to decrease.

The Pearson correlation coefficient for all samples was calculated to assess the linear relationship between IP and CFU/g. The correlation coefficients were found to be 0.950 (significant for $p < 0.01$), 0.791 (significant for $p < 0.05$), 0.853 (significant for $p < 0.01$), and 0.559 (significant for $p < 0.05$) for samples S1, S2, S3, and S4, respectively. Therefore, in all cases, a moderate-high correlation between the number of CFU and the IP was found.
suggesting a possible explanation of the increased oxidative stability of tzatziki salad due to the presence of microorganisms.

In the case that the microbial content of the salad is responsible for the oxidative stability of the product, it would be logical that the more microorganisms present in the sample, the higher the oxidative stability. Therefore, by suppressing the microbial flora of tzatziki, the oxidative stability would also decrease. To further examine this notion and the relation between microbial content and oxidative stability, two more tzatziki samples were prepared (M1 and M2), so as to determine the effect of garlic, cucumber, and preservatives on oxidative stability via microbial population. Moreover, this recipe better resembles the commercially available samples, and as such, a better overview of both homemade and commercially available samples is gained. Garlic is known to contain natural bioactive compounds (e.g., allicin) with antibacterial activity [23,24]. This is also the case with cucumber, which in addition can enzymatically form secondary bioactive compounds with various activities [1]. Moreover, sodium benzoate and potassium sorbate are well-known food additives, used to decrease the microbial population. Therefore, the addition of the above in sample M1 is expected to decrease the number of microorganisms compared to sample M2. The results for the two samples are given in Figure 2. The results validated our hypothesis that the addition of the antimicrobial ingredients would result in a faster reduction in the microbial population. Moreover, it can be seen that the resistance to oxidation of the lipid phase appeared to be significantly \( (p < 0.05) \) reduced after 16 days of storage for both samples. However, the oxidative stability of sample M2 declined to a lower extent compared to sample M1. Additionally, due to the nature of artificial preservatives used (sodium benzoate and potassium sorbate), it cannot be concluded that they act as pro-oxidants. Since the only difference between the two samples, apart from the existence or not of preservatives, garlic, and cucumber, was the microbial population, a correlation analysis was again carried out. The correlation coefficients were found to be 0.988 (significant for \( p < 0.01) \) and 0.984 (significant for \( p < 0.01) \) for samples M1 and M2, respectively. Again, the correlation was found to be high, strengthening our hypothesis that the oxidative stability of tzatziki is related to the microbial population, acting as a free radical and/or oxygen scavenger.

The microbial content of tzatziki derives from the yogurt, which is a fermented dairy product. The fermentation happens with the addition of a starter culture containing LAB such as *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* [25]. LAB are acknowledged for their health benefits, as they are normal inhabitants of the human gastrointestinal tract. They have many metabolic pathways through which they can tolerate and adapt to oxidative stress. Considering that they are facultative anaerobes and metabolize carbohydrates, LAB implement oxidation and reduction reactions. In some of these reactions, molecular oxygen is used as a substrate [26]. According to Repine et al. [27], the presence of oxygen can produce \( \text{O}_2 \) toxic intermediates such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. The above-mentioned intermediates are formed intracellularly, as well. As indicated by Saffari and Sadrzadeh [28], reactive oxygen species and hydroxyl radicals are highly reactive. Therefore, they can react with various molecules, resulting, among other things, in conformation alterations in proteins, causing lipid peroxidation, as well as damage to cellular components, ultimately leading to cellular death. In order to overcome oxidative stress, LAB possess a sensitive response and strong antioxidant activity in order to remove the oxidant compounds and repair the damage induced [26,29]. Normally, oxygen is consumed by various oxidases and produces hydrogen peroxide, which then reacts with iron ions, producing free radicals that induce oxidative damage. However, LAB have enzymes able to degrade hydrogen peroxide, such as pseudocatalase, thus reducing the concentration of hydrogen peroxide and the subsequent damage to proteins, lipids, and DNA [30]. There are also other antioxidant defense systems that LAB utilize to protect themselves from oxidative damage, resulting in an overall protection of the host organism. According to Feng et al. [30], LAB can act as radical scavengers, enzyme regulators, metal ion chelators, and microbial flora regulators.
Therefore, they have multiple forms of antioxidant activity that can be used in favor of the host organism. In our case, instead of the gastrointestinal tract, LAB are contained in the tzatziki salad. Therefore, their antioxidant potential can be used to protect tzatziki from oxidation, as already proved. This property can have a major impact on food technology, since LAB have not been used, to the best of our knowledge, for the oxidative production of food products. So far, LAB have been used as probiotics in order to promote health of consumers. However, according to our study, it is evident that the benefits of LAB for consumers begin from the protection of the food product from oxidation, thus limiting the degradation of various components.

Figure 2. Induction periods (IP) and colony-forming units (CFUs) per g of lipid phase of tzatziki samples M1 and M2 during 16 days of storage.

4. Conclusions

According to the results of this study, tzatziki is an oxidative-stable product. The recipe used for its production can have a significant impact on its oxidative stability. The use of set sheep yogurt and olive oil results in greater resistance to oxidation compared to traditional Greek-style sheep yogurt and soybean oil. This type of yogurt was found to differ in microbial content, and as such, affected the oxidative stability. Despite the content in tzatziki of tocophersols that exhibit antioxidant activity, no correlation was found between the two. Interestingly, a strong correlation between the number of microbes in tzatziki and its oxidative stability was recorded. The use of preservatives, garlic, and cucumber reduces the oxidation resistance due to a decrease in the microbial population. Therefore, it can be concluded that the microbial flora of tzatziki has antioxidant activity, protecting its lipid phase against oxidation. This property can be reaped for the preparation of tzatziki salads with even more increased oxidative stability. Moreover, the results from this study can serve as a benchmark for future studies that will examine the effect of food microorganisms and their oxidative stability.

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