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Preservation of Traditional Cheese Wagashi Using Essential Oils: Impact on Microbiological, Physico-chemical and Sensorial Characteristics

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Authors' contributions

This work was carried out in collaboration between all authors. Author PS designed the study. Author IY performed the statistical analysis. Authors PS, CB, GH, SDO and EH wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors PS, CB, GH, SDO, EH, PA, IY, DS and SF managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The high demand for food products without chemical additives has increased the use of natural preservatives like essential oils in food processing. The present study aimed to assess the impact of selected essential oils on the microbiological, physico-chemical and sensorial characteristics of preserved traditional cheese wagashi in order to improve its quality.

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Materials and Methods: Microbiological and physico-chemical parameters were analysed repeatedly on days 0, 7, 30 and 60 using normalized methods in wagashi samples kept at 25℃ and 4℃. Sensorial analyses were carried out once in 2 days old wagashi samples.
Results: Findings show that none of the analysed samples during the experimental period contains pathogenic microorganisms including *Salmonella* spp*, Staphylococcus aureus*, *Escherichia coli* and the spores of *Clostridium* spp. The pH of wagashi samples kept at 25℃ decreased o ver time while their acidity, as well as the thiobarbituric acid index increased. The trend of these physico-chemical parameters was similar at 25℃ as at 4℃; however the fluctuations were quite lower for the pH and the acidity at 4℃ than at 25℃. For the sensorial quality of the initial products, the sample treated with the essential oil of *Cymbopogon citratus* was well appreciated by the tasters who mentioned that it was of good quality followed by the sample of wagashi treated with the essential oil of *Pimenta racemosa* qualified of fairly good. Samples treated with the essential oils of *Ocimum gratissimum* and *Syzygium aromaticum* were of acceptable quality.
Conclusion: This study revealed that the application of essential oils of *Cymbopogon citratus* and *Pimenta racemosa* in replacement of chemical additives constitutes a reliable alternative for the preservation of wagashi. However, a thorough study on the biochemical characterization of the samples is necessary to appreciate the quality of the elaborated products.

Keywords: Cheese wagashi; preservation; essential oils; chemical additive; Benin.

1. INTRODUCTION

In Benin, milk is transformed into various derived products such as yogurt, curdled milk and especially wagashi, a cheese prepared through an artisanal process mastered by the Peuhl ethnic group. Wagashi is the most widespread dairy product and the most consumed in the country [1,2]. This cheese, often used in replacement of meat and fish in different dishes, constitutes an important source of animal proteins of excellent nutritional values mainly for low income populations [2,3]. It is therefore used to combat malnutrition notably protein-energy deficiency highly found in developing countries. The low lactose content of wagashi makes it a recommended food for people with lactose intolerance [4]. Despite its great economic and nutritional importance, the preservation of wagashi encounters a number of constraints including the ineffectiveness of traditional preservation methods leading to a rapid deterioration of the product. The storage of wagashi into the open air and the absence of suitable packaging increase the risks of microbial and chemical contaminations, limiting thereby the abilities for preservation, transportation and commercialisation of wagashi in the national, regional and international markets [3]. These challenges related to the preservation and the valorisation of wagashi call for the development of alternative preservation techniques that suit the socioeconomic and environmental context of the country. In order to prolong the length of preservation of wagashi, several researchers have worked on its stabilization by chemical treatment and packaging. These studies showed that the treatment of wagashi with 10% propionic acid and 0.1% sorbic acid prolonged its shelf life but adulterated the organoleptic quality of the product and affected its acceptability to consumers [5]. Moreover, the use of chemical preservatives could lead to the occurrence of chemical residues potentially harmful to consumer's health [6]. Kèkè et al. [7] used a freeze-dried strain of *Lactobacillus plantarum* for the preservation of wagashi and concluded that the obtained product was fermented and not well appreciated by tasters. All these studies could not significantly improve the general quality of wagashi. Therefore, new research perspectives are needed on this theme to establish effective preservation practices capable of ensuring the safety and the commercial quality of wagashi that can be transferred to other stakeholders in the field. Investigations on the use of plant extracts for the preservation of wagashi are necessary because of the increasing demand of consumers for safe food products prepared without chemical additives. Additionally, there is an increased interest for the use of natural antimicrobial substances such as the Generally Recognized as Safe (GRAS) [6] essential oils for food preservation [8]. In view of this, Sessou [9] initiated studies on the use of essential oils in the preservation of wagashi. This author reported that the essential oils of *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* possess strong antifungal properties and can be used for seasoning and preservation of wagashi in replacement of chemical additives. The present study was

undertaken as a continuation of the previous studies of this author and aimed to assess the impact of the essential oils of *Syzygium aromaticum*, *Pimenta racemosa*, *Ocimum gratissimum* and *Cymbopogon citratus* on the microbiological, physico-chemical and sensorial characteristics of wagashi.

2. MATERIALS AND METHODS

2.1 Production of Wagashi

At this stage, improved wagashi were produced with the studied essential oils. Samples were prepared using a modified technological diagram adopted from Dossou et al. [5]. The coagulum was obtained from a mixture of one litre of milk and 10 g of the leaves of *Calotropis procera* cooked together during 30 minutes at an internal temperature of 91.5 to 95.5°C and then drained. Thereafter, 1000 ppm of essential oil being the highest minimal inhibitory concentration of the extracts on fungal isolates of wagashi [9] was added to 100 g of the cheese. A total of six lots of 10 wagashi samples were made. These include four experimental groups treated with the essential oils of *Pimenta racemosa*, *Syzygium aromaticum, Ocimum gratissimum* and *Cymbopogon citratus* respectively and two control groups constituted of 1000 ppm of sorbic acid (positive control) based on the studies of Aworth and Egounlety [10] and wagashi samples without additive that served as negative control.

2.2 The Used Essential Oils

The essential oils used in this study were those of *Pimenta racemosa, Syzygium aromaticum, Ocimum gratissimum* and *Cymbopogon citratus*. These essential oils were extracted by hydrodistillation and previously analysed by Sessou et al. [11] using gas chromatography and gas chromatography coupled with mass spectrometry.

2.3 Chemical Additives

Sorbic acid (Prod 30241, Nutrichem, Nigeria) was the chemical preservative used in this study. This product served as positive control with respect to the studies of Aworth and Egounlety [10], who used it for the stabilization by chemical treatment of wagashi.

2.4 Evaluation of the Microbiological, Physico-chemical and Sensorial Qualities of Wagashi Treated with the Essential Oils

The microbiological and physico-chemical analyses were carried out repeatedly on days 0, 7, 30 and 60 in wagashi samples kept at 25°C and 4°C. The sensorial qualities were analysed only once on 2 days old wagashi samples.

2.4.1 Physico-chemical analyses

The physico-chemical analyses concerned the determination of water activity and the water content of the samples on the first day of production, as well as the pH and the acidity. The level of lipids' oxidization was also assessed in wagashi samples under preservation throughout the study period via the determination of the thiobarbituric acid index. Methods described by Anihouvi et al. [12] were used to determine the pH and the acidity of the samples. Twenty (20) g of wagashi sample was mixed with 80 ml of distilled water using a stomacher bag and the pH was thereafter measured using a digital pH-meter (Inolab pH 730 WTW 82362 Wellheims, Germany). Furthermore, to 5 ml of the previous suspension used to determine the pH was added 75 ml of distilled water and the titre of the mixture was determined at 0.1 N of sodium hydroxide (NaOH) using phenolphtalein as indicator. The volume of NaOH used was recorded and 1 ml of NaOH was considered equivalent to 9 mg of lactic acid.

The thiobarbituric acid (TBA) index was determined using the spectrophotometric extraction method following the protocol described by Tajik et al. [13]. 10 g of wagashi was homogenized with 35 ml of cold extraction solution (4°C) containing 4% of perchloric acid and 1 ml of Butyl Hydroxyl-Toluène (1 mg/ml) and centrifuged at 10000 rpm for 1 minute. The mixture was filtered using Whatman n°1 filter paper in a 50 ml flask. The filtrate was adjusted to 50 ml with 4% perchloric acid then 5 ml of aliquot of the filtrate was mixed with 5 ml of TBA (0.02 M). The mixture was vortexed then incubated in water bath at 100°C for 60 minutes to develop the complex malonaldehyde-TBA. The absorbance was measured at 532 nm after having cooled the solution with tap water for 10 minutes. The TBA value was expressed in mg of malonaldehyde per kg of sample.

The water content of the samples on day 0 was determined using the AOAC method [14] reported by Gnansounou et al. [15] that consists of keeping the sample in a steam-room at 104℃ for 24 hours. The water activity of the samples on the day of production was determined using an a_w-meter following the method described by Kpoclou et al. [16].

2.4.2 Microbiological analyses

The analysed microbiological parameters were the enumeration of total microbial count, lactic acid bacteria count and magnitude of *Staphylococcus aureus, Escherichia coli, Clostridium* spp, yeasts and moulds in the samples together with the detection of *Salmonella* spp*.* The total flora was counted using the standard method NF IN ISO 6222: 1999 on Plate count agar at 30℃ for 72 hours, while yeasts and moulds were enumerated according to ISO 7954:1987 method on Sabouraud media mixed with chloramphenicol at 25℃ for 5 days. The lactic flora was counted on Man Rogosa Sharp media in microaerophily. *Staphylococcus aureus* was counted according to the NF IN ISO 6888 - 1:1999 standards on Baird Parker culture medium at 37℃. Enumeration of *E. coli* was done using NF ISO 16649-2 method on Rapid *E. coli* media at 44℃. Detection of *Salmonella* spp was carried out following the NF IN ISO 6579:2002 standards. The spores of *Clostridium* were counted in test tube using Tryptone Sulfite Neomycin medium according to the XP V 08-061:1996 standards.

2.4.3 Sensorial analyses

Parameters such as texture, colour, odour, appearance and the taste (bitter, salty, pricking, acidic, acrid, sweet) were assessed according to the method described by Pezeshk et al. [17]. This analysis consisted of appreciating the organoleptic acceptability of the wagashi samples on the second day of production by a panel of 50 tasters and wagashi consumers previously trained for the test. The wagashi samples were judged by the evaluators based on the following criteria: Excellent (6), very good (5), good (4), fairly good (3) acceptable (2) and mediocre (1). The pieces of cheeses that were used for the tests were boiled separately in a pan without seasoning and salt for about 5 minutes at an internal temperature of 95℃. Furthermore, the colour and the odour of non-boiled samples were appreciated by the judges.

2.5 Statistical Analyses

The recorded data were stored in Excel database and analysed with SAS [18] software. The physico-chimical and microbiological parameters of wagashi samples were determined per time during preservation. The data obtained for each of these parameters were analysed with the same software SAS [18]. The procedure of Generalized Linear Models (Proc GLM) was used for the analysis of variance. The types of preservatives (essential oils and chemical additive) and the temperature of storage were considered as sources of variation. Means were calculated per storage temperature and type of product for the different parameters. The significance of the effects of the preservatives or the temperature was determined using F test. Means and standard deviations of the physico-chemical and microbiological parameters were calculated and compared two by two using the student t test.

3. RESULTS AND DISCUSSION

3.1 Evolution of the Physico-chemical and Microbiological Characteristics of the Treated Wagashi Samples per Time and Temperature of Preservation

3.1.1 Physico-chemical characteristics of wagashi samples

Table 1 shows the results of the physico-chemical parameters that are water content and water activity of the wagashi samples on day 0 of production. The results revealed that the water contents of the samples varied between 63.068±0.604% and 66.817±2.717% while the water activity of the same samples varied from 0.895±0.007 to 0.914±0.000. Moreover, the sample treated with the essential oil of *Ocimum gratissimum* had a lower average water content ($p < 0.05$) as compared to the other samples. The different recorded water contents of the samples are similar to those of Kora [19] reported by Sacramento [4] that varied from 65.23 to 66.13. The lowest water activity was recorded from the sample treated with the essential oil of *Ocimum gratissimum*. Samples of the negative control group had the highest water activity as compared to the other samples. Statistical analyses demonstrated that there was a significant difference ($P<0.05$) between the values of water activity of the samples. The

decrease of water activity in samples treated with preservatives especially with the essential oil of *Ocimum gratissimum* compared to the untreated wagashi samples testifies a leakage of free water in the treated samples due to the molecules present in the preservatives. In other words, the decrease of water activity especially with the essential oil of *Ocimum gratissimum* could be explained by the fact that the compounds present in the essential oils act on the availability of residual water and concentrate the dried matter. The reduction of free water in the wagashi samples under the influence of essential oils and sorbic acid could be a hurdle to the growth of some fragile microbial strains that need a high water content to grow. High level of free water constitutes a very important factor in food preservation because it enhances the multiplication of microorganisms and reduces the shelf life of the food [16]. According to Prescott et al. [20], low water activity in food contributes to the inhibition of the growth of pathogenic microorganisms such as *Salmonella* spp*, Escherichia coli, Clostridium botulinum* and *Clostridium perfringens* which require minimal water activities of 0.95, 0.94, 0.95 and 0.95, respectively [21].

Results of the pH, the acidity, as well as the thiobarbituric acid index that informs on lipids' oxidization of the different samples during their preservation are displayed in Tables 2 to 5. The pH of wagashi samples ranged between 4.47 and 6.70 for samples kept at 25℃ and from 5.03 to 6.70 for samples kept at 4℃. The acidity varied from 1.8 to 49.95%0 of lactic acid for samples kept at 25℃ and 1.8 to 11.60%0 at 4℃. The concentration of malondialdehyde (MDA), detected through the thiobarbituric acid index (TBA index) of the wagashi samples varied between 0.484 and 6.801 mg of malonaldehyde/ kg of wagashi for samples kept at 4℃ and from 0.391 to 5.080 mg of malonaldehyde/kg of wagashi for the samples kept at 25℃. All samples had a TBA index lower than 1 mg of malonaldehyde/kg of wagashi during the first two weeks of preservation at the two temperatures (25℃ and 4℃) except the sample that was treated with sorbic acid and kept at 25℃ with a TBA index of 1.321 mg of malonaldehyde/kg of wagashi. Samples treated respectively with the essential oils of *Cymbopogon citratus, Syzygium aromaticum* and sorbic acid and kept at 4℃ were decreasingly those that are more exposed to lipid oxidization reactions with very high TBA indexes on the 60th day of preservation. This explains their advanced level of deterioration and the limit of the activity of these essential oils as time goes. The wagashi sample treated with the essential oil of *Pimenta* racemosa was the most spoiled sample at 25℃ with a high concentration of malonaldehyde (5.080 mg of malonaldehyde/ kg of wagashi) on the 60th day of storage. Furthermore, the analyses revealed that the pH of the samples decreased over time. This decrease of pH was more pronounced in samples kept at 25℃ and could be explained by metabolic reactions of lactobacilli that are present in the cheese and transform the lactose of this latter into lactic acid. By analogy, the acidity of the samples kept at 25℃ increased with the time and correlated positively with the pH of these samples. The trend of these physico-chemical parameters was similar at 25℃ as at 4℃; however the fluctuations were quite lower for the pH and the acidity at 4℃ than at 25℃. The pH and the TBA index of refrigerated samples varied very weakly during the first two weeks of preservation. Overall, samples treated with the four essential oils and kept at 4℃ conserved approximatively their physico-chemical quality for two weeks, whereas those kept at 25℃ had their acidity considerably increased during this period. The results of pH and acidity of this study are in conformity with those of Kèkè et al. [22] who reported that wagashi samples kept at ambient temperature had a lower pH and higher acidity in percentage of lactic acid than those that were refrigerated. The pH of the samples of the current study on the day of production and the ones of those refrigerated during the first two weeks are similar to those reported by Sacramento [4] that varied between 6.4 and 6.5. The decrease of pH in the samples is in agreement with the results of Salaka et al. [23] who observed a reduction of pH in beef samples, a food rich in protein like wagashi, during the preservation period. Moreover, this decrease of pH especially in samples kept at 25℃ can be attributed to the predominance of mesophilic and psychrotrophic lactic acid bacteria that could colonised the wagashi at the time of preservation [23]. This predominance of lactobacilli leading to the production of lactic acid will decrease the pH of the cheese and thereby increase its acidity. According to Mahmoudi et al. [24], lactobacilli represent a serious problem for products that are packaged under vacuum in the sense that they can multiply and produce different metabolites. The increased acidity of wagashi samples kept at ambient temperature has a negative impact on these samples since wagashi is not normally an acidic product according to the consumers'

quality criteria [2,4]. Therefore, wagashi kept at ambient temperature is of non-satisfactory quality after a week as opposed to those kept at 4℃ and the discriminative parameters are the pH and the acidity. The increase of TBA index in the samples can be attributed to the increase of free radicals in these samples leading to their spoilage that was observed at ambient temperature after one week of preservation by a putrid smell. Furthermore, the increase of TBA index at ambient temperature, as well as at 4℃ shows that the reaction of lipids' oxidization does not depend on the storage temperature. Ruiz-Capillas and Moral [25] reported that unlike microbial and enzymatic reactions, low temperature and modified preservation atmosphere do not influence significantly lipid oxidization reactions in food. Despite the products being stored under vacuum, this elevation of the TBA index at both storage temperatures can be attributed to the deterioration of the quality of the vacuum due to the presence of food exudate that could have modified the barrier properties of the oxygen of the bag [26]. The high concentrations of malonaldehydes in samples that are treated with preservatives can also be achieved by overestimating the values. In fact, substances other than malonaldehydes (MDA) like aromatic aldehydes that constitute the flavour profile of various fruits and essential oils, can react with thiobarbituric acid in hot acidic environment to generate a complex detectable by spectro-photometry at 532 nm [27,28,29]. The high concentration of MDA (>1) in the samples after two weeks of preservation is dangerous to the quality of the products and the health of consumers. In fact, MDA is one of the many electrophile reactive species that cause stress in cells and form advanced derivatives of glycation that are implicated in some degenerative diseases like cancer and kidney failure. Moreover, MDA can produce some derivatives with free amino acids and a lot more with proteins and this can lead to a thorough change of their biochemical properties [30].

3.1.2 Microbiological characteristics of wagashi samples

Microbiological analyses showed that no pathogen including *Salmonella* spp*, Staphylococcus aureus, Escherichia coli* and *Clostridium* spp were found in the samples during the experimentation period. On the day of production, the total microbial count was between 0.430 x 10^2 cfu/g and 268 x 10^2 cfu/g and the lactic flora varied between 0.170 x 10^2 cfu/g and 1.55 x 10^2 cfu/g. The level of contamination by yeasts and moulds varied from 0 to 10^1 cfu/g. Overall, all these microbial loads increased over time. For samples kept at 4℃, the total flora varied from 0.43 x 10^2 cfu/g to 37.50 x 10^2 cfu/g, the lactic flora from 0.170 x 10^2 cfu/g to 35.650 x 10^2 cfu/g and the fungal flora from 0 to 53.000 x 10^1 cfu/g. However, samples that were kept at 25℃ had higher contamination level as compared to those refrigerated. At 25℃, the total flora varied from 0.430 x 10^2 cfu/g to 268 x 10^2 cfu/g, the lactic flora from 0.170 x 10^2 cfu/g to 252 x 10^2 cfu/g and the fungal flora from 0 to 21 x 10^1 cfu/g. The highest contaminations at 4℃ and 25℃ occurred in the negative control group containing untreated samples. The increase of microbial loads especially for moulds in wagashi samples stored at ambient temperature can distort their nutritional and organoleptic quality which can provoke the loss of commercial value. The absence of pathogenic germs in the samples is probably due to the time/temperature ratio of cooking. In fact, most of the targeted microorganisms are easily destroyed by pasteurization, but these samples were almost sterilised during the thorough cooking. According to Avens et al. [31],

Table 1. Water activity and water content of wagashi samples

Samples	Water activity			Water content		
	N	Mean	Standard deviation	N	Mean	Standard deviation
F1	2	0.895c	0.007	3	63.068b	0.604
F2	2	0.906b	0.001	3	65.932ab	1.88
F3	2	0.897b	0.001	3	66.687ab	4.597
F4	2	0.904b	0.001	3	65.632a	0.963
F5	2	0.904b	0.001	3	66.548a	0.626
F6	2	0.914a	0.000	3	66.817ab	2.717
Significance Test		*		*		

Values in the same column followed by different letters are significantly different at P<0.05. **** :*** *significant at P<0.05, F1 : wagashi +EO$_{Ocimum\ gratissimum}$; F2 : wagashi +EO$_{Cymbopogon\ citratus}$; F3 : wagashi +EO$_{Pimenta\ racemosa}$; F4 : wagashi +EO$_{Syzygium\ aromaticum}$; F5 : wagashi + Sorbic acid ; F6 : untreated wagashi, EO: Essential oil*

Table 2. Physico-chemical and microbiological parameters of wagashi samples kept at 25℃

Day 0 (25℃)														
Treatment	**N**	**TNB (10^2 cfu/g)**		**LAB (10^2 cfu/g)**		**Moulds (10^1 cfu/g)**		**Yeasts (10^1 cfu/g)**		**pH**		**Acidity (%O)**		**TBA index**	
		Mean	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**
Cheese So. ac	2	2.360bc	1.301	1.310a	0.042	1.000a	0.000	0.000b	0.000	6.591a	0.110	2.700a	0.000	0.667c	0.000
Cheese white	2	6.600a	0.282	1.550a	0.636	0.000b	0.000	1.000a	0.000	6.706a	0.030	1.800b	0.000	0.773b	0.002
Cheese Cy.c	2	0.430c	0.410	0.170c	0.042	0.000b	0.000	0.000b	0.000	6.608a	0.188	1.800b	0.000	0.627d	0.001
Cheese Oc.gr	2	2.000b	1.414	0.220bc	0.085	0.000b	0.000	0.000b	0.000	6.559a	0.169	1.800b	0.000	0.901a	0.070
Cheese Pi. ra	2	1.190bc	0.438	0.880ab	0.452	0.000b	0.000	1.000a	0.000	6.622a	0.144	1.800b	0.000	0.618e	0.001
Cheese Sy. ar	2	2.110bc	1.117	1.150a	0.212	0.000b	0.000	0.000b	0.000	6.596a	0.237	1.800b	0.000	0.572f	0.003
Significance test		**		*		***		***		NS		***		***	
Day 14 (25℃)															
Treatment	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**
Cheese So. ac	2	7.800b	0.848	4.200bc	0.141	1.000a	0.000	115.000ab	21.213	4.848f	0.001	14.400a	0.000	1.321a	0.078
Cheese white	2	62.200a	18.384	23.000a	2.828	0.000b	0.000	138.000a	14.142	5.428b	0.004	14.850a	0.636	0.417cd	0.005
Cheese Cy.c	2	6.050b	0.636	2.250c	0.353	0.000b	0.000	115.000ab	7.071	5.153d	0.011	8.100c	0.000	0.568b	0.012
Cheese Oc.gr	2	6.400b	0.990	4.900bc	0.424	0.000b	0.000	1.000c	0.000	5.547a	0.002	8.550c	0.636	0.485bc	0.004
Cheese Pi. ra	2	9.340b	0.650	6.350b	1.202	1.000a	0.000	99.500b	14.849	4.891e	0.000	11.250b	0.636	0.510bc	0.054
Cheese Sy. ar	2	7.800b	0.707	4.250bc	0.071	0.000b	0.000	26.500c	0.707	5.342c	0.013	10.800b	0.000	0.391d	0.002
Significance test		**		***		***		***		***		***		***	

Values of the same column followed by different letters are significantly different at P<0.05 ****:*** *significant at P<0.05* ***** :*** *significant at P<0.01,* ****** :*** *significant at P<0.001, LAB: Lactic Acid Bacteria, TNB: Total Number of Bacteria, TBA: Thiobarbituric Acid, Cheese Ac. so : wagashi + Sorbic acid; Cheese white : untreated wagashi; Cheese Cy c : wagashi +essential oil of Cymbopogon citratus ; Cheese Oc gr. : wagashi + essential oil of Ocimum gratissimum; Cheese Pi ra. : wagashi + essential oil of Pimenta racemosa, Cheese Sy. ar : wagashi + essential oil of Syzygium aromaticum*

Table 3. Physico-chemical and microbiological parameters of wagashi samples kept at 25℃

Day 30 (25℃)															
Treatment	**N**	**TNB (10^2 cfu/g)**		**LAB (10^2 cfu/g)**		**Moulds (10^1 cfu/g)**		**Yeasts (10^1 cfu/g)**		**pH**		**Acidity (%O)**		**TBA index**	
		Mean	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Treatment**	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**
Cheese So ac	2	26.350b	0.212	5.250d	0.071	2.000b	0.000	17.000bc	1.414	4.885d	0.007	34.200a	0.000	1.343c	0.021
Cheese white	2	90.000a	16.970	40.000a	2.830	4.000a	1.414	124.500a	7.778	5.118c	0.022	18.000d	1.273	0.971e	0.007
Cheese Cy.c	2	9.550b	0.071	8.600c	0.850	1.000c	0.000	11.500c	0.710	4.681e	0.010	28.800b	1.273	1.359c	0.008
Cheese Ocgr	2	10.750b	1.768	9.450c	0.353	1.000c	0.000	21.500b	0.710	5.267b	0.013	27.000b	0.000	2.260b	0.018
Cheese Pi Ra	2	11.650b	3.040	9.050c	0.353	4.500a	0.710	18.000bc	1.414	6.193a	0.001	12.600e	2.545	2.463a	0.003
Chees Sy.Ar	2	14.800b	0.710	14.300b	0.424	1.000c	0.000	18.500bc	0.710	4.475f	0.010	23.850c	0.636	1.257d	0.004
Significance test		***		***		**		***		***		***		***	
Day 60 (25℃)															
Treatment	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**
Cheese So ac	2	89.500c	12.020	50.150d	6.859	9.000b	1.414	15.500d	3.535	5.798b	0.003	49.950a	1.909	1.647e	0.008
Chees white	2	268.000a	56.568	252.000a	11.318	1.500c	0.710	49.000a	1.414	4.670d	0.000	24.300c	1.273	2.551b	0.020
Cheese Cy.c	2	109.300b	14.566	46.100d	4.384	1.000c	0.000	7.000e	1.414	4.480e	0.000	26.550c	4.454	1.996d	0.021
Cheese Ocgr	2	158.000b	22.627	109.550b	13.788	1.000c	0.000	8.500e	0.710	4.700c	0.014	25.200c	1.273	5.080a	0.071
Cheese Pi Ra	2	111.000b	11.314	98.650b	0.919	21.000a	4.242	43.500b	2.121	6.475a	0.007	13.050d	0.636	2.424c	0.023
Cheese SyAr	2	103.650bc	15.061	74.450c	0.636	1.000c	0.000	38.000c	2.828	4.480e	0.000	32.400b	1.273	1.582e	0.024
Significance test		**		***		***		***		***		***		***	

Values of the same column followed by different letters are significantly different at P<0.05 ****:*** *significant at P<0.05* ***** :*** *significant at P<0.01,* ****** :*** *significant at P<0.001, LAB: Lactic Acid Bacteria, TNB: Total Number of Bacteria, TBA: Thiobarbituric Acid, Cheese Ac. so : wagashi + Sorbic acid; Cheese white : untreated wagashi; Cheese Cy c : wagashi +essential oil of Cymbopogon citratus ; Cheese Oc gr. : wagashi + essential oil of Ocimum gratissimum; Cheese Pi ra. : wagashi + essential oil of Pimenta racemosa, Cheese Sy. ar : wagashi + essential oil of Syzygium aromaticum*

Table 4. Physico-chemical and microbiological parameters of wagashi samples kept at 4℃

Day 0 (4℃)															
Treatment	**N**	**TNB (10^2 cfu/g)**		**LAB (10^2 cfu/g)**		**Moulds (10^1 cfu/g)**		**Yeasts (10^1 cfu/g)**		**pH**		**Acidity (%O)**		**TBA index**	
		Mean	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Treatment**	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**
Cheese So Ac	2	2.360bc	1.301	1.310a	0.042	1.000a	0.000	0.000b	0.000	6.591a	0.110	2.700a	0.000	0.667c	0.000
Cheese white	2	6.600a	0.282	1.550a	0.636	0.000b	0.000	1.000a	0.000	6.706a	0.030	1.800b	0.000	0.773b	0.002
Cheese Cy.c	2	0.430c	0.410	0.170c	0.042	0.000b	0.000	0.000b	0.000	6.608a	0.188	1.800b	0.000	0.627d	0.001
Cheese Oc.gr	2	2.000b	1.414	0.220bc	0.085	0.000b	0.000	0.000b	0.000	6.559a	0.169	1.800b	0.000	0.901a	0.070
Cheese Pi. Ra	2	1.190bc	0.438	0.880ab	0.452	0.000b	0.000	1.000a	0.000	6.622a	0.144	1.800b	0.000	0.618e	0.001
Cheese Sy. Ar	2	2.110bc	1.117	1.150a	0.212	0.000b	0.000	0.000b	0.000	6.596a	0.237	1.800b	0.000	0.572f	0.003
Significance Test		**		*		***		***		NS		***		***	
Day (4℃)															
Treatment	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**
Cheese So Ac	2	4.650b	2.192	2.300b	0.141	1.000a	0.000	1.000b	0.000	6.390a	0.013	3.600a	0.000	0.716a	0.002
Cheese white	2	16.750a	2.899	12.000a	1.414	0.000b	0.000	2.500a	0.710	6.345a	0.024	3.600b	0.000	0.647b	0.020
Cheese Cy.c	2	5.150b	1.343	2.150	0.495	0.000b	0.000	0.000c	0.000	6.430a	0.087	2.700b	0.000	0.508d	0.011
Cheese Oc.gr	2	5.150b	1.061	3.350b	0.071	0.000b	0.000	0.000c	0.000	6.125a	0.242	3.600b	0.000	0.714a	0.032
Cheese Pi. Ra	2	2.150b	0.495	1.850b	0.495	0.000b	0.000	1.000b	0.000	6.474a	0.010	3.150b	0.636	0.563c	0.001
Cheese Sy. Ar	2	5.900b	0.990	2.550b	0.212	0.000b	0.000	3.000a	0.000	6.495a	0.064	2.700b	0.000	0.484d	0.017
Significance test		**		***		***		***		NS		*		***	

Values of the same column followed by different letters are significantly different at P<0.05 ****:*** *significant at P<0.05* ***** :*** *significant at P<0.01,* ****** :*** *significant at P<0.001, LAB: Lactic Acid Bacteria, TNB: Total Number of Bacteria, TBA: Thiobarbituric Acid, Cheese Ac. so : wagashi + Sorbic acid; Cheese white : untreated wagashi; Cheese Cy c : wagashi +essential oil of Cymbopogon citratus ; Cheese Oc gr. : wagashi + essential oil of Ocimum gratissimum; Cheese Pi ra. : wagashi + essential oil of Pimenta racemosa, Cheese Sy. ar : wagashi + essential oil of Syzygium aromaticum*

Table 5. Physico-chemical and microbiological parameters of wagashi samples kept at 4℃

Day 30 (4℃)															
Treatment	**N**	**TNB (10^2 cfu/g)**		**LAB (10^2 cfu/g)**		**Moulds (10^1 cfu/g)**		**Yeasts (10^1 cfu/g)**		**pH**		**Acidity (%O)**		**TBA index**	
		Mean	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Treatment**	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**
Chees So. ac	2	7.500b	0.920	2.500bd	1.273	1.000a	0.000	11.000c	1.414	6.306b	0.004	5.400c	0.000	2.559c	0.002
Cheese white	2	21.950a	2.616	18.500a	0.710	0.000b	0.000	1.500d	0.710	6.086d	0.013	5.400c	0.000	2.890b	0.036
Cheese Cy.c	2	9.000b	0.282	1.800cd	0.283	1.000a	0.000	12.500c	0.710	5.568f	0.006	3.600d	1.273	2.909b	0.026
Cheese Oc.gr	2	7.050bc	0.353	1.100d	0.141	1.000a	0.000	49.000a	1.414	5.958e	0.006	7.200b	0.000	2.142e	0.012
Cheese Pi. ra	2	3.700c	1.131	3.350bc	0.353	0.000b	0.000	53.000a	4.243	6.170c	0.011	6.300bc	0.000	3.132a	0.025
Chees Sy. ar	2	7.200bc	2.262	3.450b	0.353	0.000b	0.000	30.500b	0.710	6.394a	0.007	11.600a	1.131	2.433d	0.001
Significance test		***		***		**		***		***		***		***	
Day 60 (4℃)															
Treatment	**N**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**	**Mean**	**Std dev**
Chese So. ac	2	32.000a	8.485	7.800bc	0.566	1.000a	0.000	1.000c	0.000	6.215b	0.049	7.650ab	1.909	3.371b	0.047
Cheese white	2	37.500a	7.778	35.650a	6.151	1.000a	0.000	1.500c	0.710	5.845c	0.064	7.200ab	0.000	1.303d	0.027
Cheese Cy.c	2	24.500ab	2.121	3.350c	0.212	0.000b	0.000	6.000b	1.414	6.090bc	0.113	5.850bc	0.636	6.801a	0.809
Cheese Oc.gr	2	16.250c	0.353	1.950c	0.353	0.000b	0.000	4.000bc	1.414	5.875c	0.148	8.550a	0.636	1.881cd	0.011
Cheese Pi. ra	2	8.750c	0.353	9.550b	0.919	0.000b	0.000	4.000bc	1.414	5.030d	0.085	9.000a	0.000	2.133c	0.000
Chees Sy. ar	2	18.300bc	1.838	8.000bc	1.414	0.000b	0.000	23.500a	2.121	6.525a	0.008	4.050c	0.636	3.884b	0.197
Significance test		**		***		***		***		***		*		***	

Values of the same column followed by different letters are significantly different at P<0.05 ****:*** *significant at P<0.05* ***** :*** *significant at P<0.01,* ****** :*** *significant at P<0.001, LAB: Lactic Acid Bacteria, TNB: Total Number of Bacteria, TBA: Thiobarbituric Acid, Cheese Ac. so : wagashi + Sorbic acid; Cheese white : untreated wagashi; Cheese Cy c : wagashi +essential oil of Cymbopogon citratus ; Cheese Oc gr. : wagashi + essential oil of Ocimum gratissimum; Cheese Pi ra. : wagashi + essential oil of Pimenta racemosa, Cheese Sy. ar : wagashi + essential oil of Syzygium aromaticum.*

over 90% of pathogenic and spoilage microorganisms are eliminated by cooking in boiling water at 95℃ for 3 minutes. Wagashi samples treated with the essential oil of *Pimenta* racemosa kept at 4℃ were less favourable to microbial growth. Samples treated with *Pimenta racemosa* had the lowest total microbial flora during the experimentation except on the day 0 where the essential oil of *Cymbopogon citratus* was the most active extract of all tested oils. These results confirm the antimicrobial activity of the essential oil of *Pimenta racemosa* and are in accordance with the findings of Fanou [32] and Sessou [9]. The microbial loads of samples treated with the four essential oils were lower than the microbial loads of the control groups. The increased growth of lactic bacteria in these samples is classical especially for samples packaged under vacuum. According to Mahmoudi et al. [24], lactobacilli represent a serious problem to products that are packed with vacuum in the sense that they multiply in these conditions and produce bad odours and sour flavours. With regards to the prescriptions of the Federation of trade and Distribution Enterprises [33], the microbiological quality of all treated samples can be regarded as satisfactory. These results are due to the good hygienic practices employed at the time of samples production and preservation and this should be taught to normal producers. Therefore, the consumption of wagashi produced in such conditions would not pose any health risk to consumers. Samples treated with the extract of *Pimenta racemosa* were microbiologically safer than the other samples at refrigeration temperature. Moreover, all samples kept at 25℃ have a lower shelf life than those preserved at 4℃; this confirms the positive influence of refrigeration on the growth of microorganisms. According to Rosset et al. [34], microbial growth is inhibited at low temperature. This effect of the cold can be explained by an inhibition of metabolic activities that are controlled by temperature dependent enzymatic systems.

3.2 Sensorial Characteristics of Wagashi Samples

This phase involved 50 judges who appreciated very well the sample treated with sorbic acid. For the essential oils, the tasting results showed that wagashi sample treated with the essential oil of *Cymbopogon citratus* was the best as this was qualified of good quality. It was followed by the sample treated with *Pimenta racemosa* judged as fairly good and those treated with the two other essential oils were of acceptable quality. However, the evaluators did not appreciate the colour of the non-boiled wagashi samples treated with the essential oil of *Cymbopogon citratus* as it was seen as unattractive. Nevertheless, the aroma of that sample was very well appreciated. An opposite observation was made by the same panel for the non-boiled sample treated with *Ocimum gratissimum*. In fact, the aroma of this sample was bad but its appearance was attractive. Overall, all these appreciations and judgements are subjective in the sense that these new products can be considered as medicinal foods.

4. CONCLUSION

The present study assessed the impact of four essential oils on the microbiological quality, physico-chemical and sensorial parameters of wagashi. The study revealed that the extracts influenced positively the microbiological quality of the treated wagashi samples. Nevertheless, these same essential oils contributed to the modification of the physico-chemical properties of the samples by increasing their thiobarbituric acid index. Overall, wagashi samples conserved their microbiological and physico-chemical quality for two weeks at 4℃ while those kept at 25℃ lost their quality and became more acidic within the same period. The study also confirmed the antimicrobial properties of the used essential oils. Moreover, the extracts of *Ocimum gratissimum* and *Syzygium aromaticum* distorted the sensorial quality of the wagashi samples, while *Cymbopogon citratus* and *Pimenta racemosa* were well appreciated. Although the sensorial quality of these samples was affected, they could still be recommended as medicinal foods with respect to the beneficial effects of these extracts on health. It is necessary to perform further metabolomics characterization of the cheese to explore production of secondary metabolites and to assess sensorial quality of the cheese considering duration of preservation and storage.

DISCLAIMER

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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