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Effect of Yoghourt Starter Culture and Nickel Oxide Nanoparticles on the Activity of Enterotoxigenic *Staphylococcus aureus* in Domiati Cheese

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Abstract: Domiati cheese is the most popular type of white soft cheese in Egypt. *Staphylococcus aureus* is a common microorganism that can easily contaminate Domiati cheese during processing and distribution. Enterotoxigenic *S. aureus* strains produce staphylococcal enterotoxins (SE) that have been involved in food poisoning outbreaks worldwide. The aim of the present study was to examine the inhibitory effect of yoghourt starter culture and nickel oxide nanoparticles (NiO NPs) on the development of the enterotoxigenic *S. aureus* together with the enterotoxin production during the manufacturing and storage of Domiati cheese. Fresh cow's milk was inoculated with *S. aureus* in a count of six log CFU/mL with the addition of either yoghourt starter culture or NiO Nps. The cytotoxicity of NiO NPs on normal human epithelial cells (HEC) was assessed using the MTT assay. In the current study, the inoculated milk was used for making Domiati cheese and the survival Weibull and log-linear models were fitted to the observed data. The obtained results showed that the mean log count of *S. aureus* decreased one week earlier by using yoghourt starter culture. Staphylococcal enterotoxin A (SEA) was identified only in the control cheese. Notably, Domiati cheese contained MIC of NiO NPs (35 µg/mL), which resulted in a significant decrease in *S. aureus* counts since at day 21 of cheese ripening it was not detected (<10 CFU/g). Overall, the current study indicated that the addition of yoghourt starter culture and NiO NPs during the processing of Domiati cheese could be useful candidates against *S. aureus* and enterotoxin production in the dairy industry.

Keywords: Domiati cheese; *S. aureus*; SEA; starter culture; nickel oxide nanoparticles; cytotoxicity

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1. Introduction

Dairy products have significant importance in our diet as milk and its derivatives can provide us with essential dietary components such as carbohydrates, proteins, fats, minerals and vitamins [1]. Hence, milk and derived products have a prominent place in the food sector. Regarding their nutritional value, cheese is one of the dairy products that has a high protein content and is also characterized by its unique flavor and aroma. In Egypt, Domiati cheese is the most important soft pickled cheese variety that is manufactured from fresh salted cow and buffalo milk through the addition of animal rennet (extracted from the fourth stomach of young calves). After processing, cheese must be stored for ripening at room temperature for six months [2]. The ripening process has a naturally effective role in the development of cheese flavor due to the production of some essential metabolites by the lactic-acid producing bacteria and molds [3]. In spite of the hygienic measures taking place at industrial levels for cheese making, some foodborne pathogens could contaminate

this kind of cheese, such as *Staphylococcus aureus* [4,5]. This microorganism has been also isolated in a wide range of cheeses and cheese products causing many recorded outbreaks in different countries worldwide [6].

S. aureus can produce staphylococcal enterotoxins (SEs) in food leading to staphylococcal food poisoning (SFP). SEs include 21 different antigenic serotypes that are previously reported, but the most common enterotoxins in food are SEA, SEB, SEC, SED and SEE which are called the classical enterotoxins that are the most common cause of staphylococcal food poisoning worldwide [7]. SEA is the most prevalent one in food in comparison to the other enterotoxins. In general, SEs can be produced in cheese when the *S. aureus* population exceeds 10^5 per gram during production and this poses a risk of toxiinfection [8]. However, the ability of *S. aureus* to produce its toxins depends on several extrinsic factors (e.g., temperature, pH, water activity and the competitive microbiota) [9]. Notably, European regulations reported that the permissible concentration of *S. aureus* in the final cheese products manufactured from heated milk must be $<1 \times 10^3$ CFU/g, and $<1.0 \times 10^5$ CFU/g for cheese products from raw milk [10]. While for Egyptian standards, the maximal limit of *S. aureus* in milk products is 100 CFU/mL [11]. Importantly, the symptoms of staphylococcal food poisoning occur in humans when as little as 20 ng of SEA is ingested in food [12]. Hence, the confirmed presence of SEs in any foodstuff is strictly prohibited in Europe [10,13].

To avoid cheese spoilage during its commercial shelf-life, different strategies are currently used, such as the addition of starter cultures during cheesemaking. It is well reported in the literature that lactic acid bacteria have an inhibitory effect against *S. aureus* growth and enterotoxins production [14,15]. In Egypt, the addition of yoghourt to Domiati cheese is a very common practice to improve the curd quality by fastening the curd formation and increasing its firmness. Hence, the present study was designed to define the effect of this ingredient on the kinetic of the enterotoxigenic *S. aureus* and enterotoxin production during manufacturing and ripening of the widely used soft cheese in Egypt.

Nanotechnology is one of the most recent methods used to increase food quality and safety [16]. Natural and engineered nanoparticles have received the attention of many researchers. Their applications are extremely close to our everyday life in agriculture, food safety, food packaging, food preservation (shelf-life extension), water purification, medicine, pharmacy and cosmetics [17]. Application of nanotechnology in the food industry leads to the development of food ingredients with high functionality such as improved physical or chemical properties and the physiological performance [18]. Consequently, it could improve the taste and nutritional value of food products and prolong their shelf-life [19]. There are widely available food products that contain nanomaterials. For instance, titanium dioxide and silica nanoparticles are now used as food additives in the food sector [20]. Additionally, in Europe, carbon black and silica dioxide nanomaterials have been already used as food contact materials [21]. Some particles were used for the destruction of microorganisms such as silver, nickel, nickel oxide, gold, zinc, metal oxides, etc. [22]. Gold nanostructures, quantum dots (QD), carbon nanotubes, and other active nanostructures have been used as sensors of microbes for food safety [23,24].

Previous studies revealed that nickel nanoparticles could exhibit excellent antimicrobial effects in comparison with other metal nanoparticles under the same condition [25]. Additionally, *S. aureus* was highly sensitive to nickel and nickel oxide nanoparticles (NiO NPs) as a prospective antibacterial agent [26,27]. Nevertheless, the toxicity of using NiO NPs in food is not fully understood; however, the use of such particles has been assessed in previous studies such as that of Haghshenas & Faraji [26] who added nickel nanoparticles to bovine milk and examined its effect on the survivability of *S. aureus*. They found that *S. aureus* was highly sensitive to Ni NPs. Because nanoparticles have never been used in cheese, the current study investigated the effect of NiO NPs on the growth and survival of enterotoxigenic *S. aureus* (harboring SEA gene) during the manufacture and storage of Domiati cheese. Besides, the inhibitory effect of yoghourt starter culture on *S. aureus* and enterotoxin production was examined during the processing and ripening of such cheese.

2. Materials and Methods

2.1. Bacterial Strain and Inoculum Preparation

Enterotoxigenic *S. aureus* strains (harboring SEA gene) were previously isolated from dairy product samples by the culture method on Baird-Parker agar and identified using conventional biochemical method and PCR. The isolates were inoculated in Brain Heart Infusion (BHI) (BBL 11407, Lansing, MI, USA) broth and incubated at 37 °C for 24 h. Before inoculation in the milk, the inoculum was washed twice in phosphate buffer saline (PBS) (Oxoid, Basingstoke, UK) and then re-suspended in skim milk.

2.2. Preparation of the Nanoparticles Suspensions and Determining the Minimum Inhibitory Concentration (MIC) of NiO NPs

Nickel oxide nanoparticles (20 nm in size) used in this study with 99.9% of purity were commercially purchased from Nan-Tech Company, Egypt. To prepare different concentrations of such particles, a solution of 10^4 µg/mL NiO NPs was used as an initial stock. Fifteen concentrations of NiO NPs (10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90 and 100 µg/mL) were examined in the present study to detect the minimum inhibitory concentration (MIC) that could inhibit *S. aureus* strains using agar well-diffusion method according to Suresh et al. [28] with some modifications. Briefly, 100 µL of the fresh bacterial culture having 10^6 CFU/mL was spread on brain heart infusion agar plates and left for 10 min to be absorbed. One-hundred µL of the nanoparticle's suspensions were poured onto the wells (8 mm). After overnight incubation at 35 ± 2 °C, the diameters of the inhibition zones were observed and measured in mm.

2.3. Assessment of Nickel Oxide Nanoparticles Cytotoxicity

This part was done at Bioassay-Cell Culture Laboratory Cell, National Research Centre (NRC), Egypt. The cell viability was assessed using MTT assay according to Mosmann [29]. Briefly, normal human epithelial cells (HEC) were batch cultured for 10 days, then seeded at a concentration of 10×10^3 cells/well in 96-well microtiter plates at 37 °C for 24 h under 5% CO_2. Media was aspirated and fresh medium (without serum) was added, then cells were incubated either alone (negative control) or with different concentrations of NiO NPs to give a final concentration of 17.5, 35, 70 and 140 ug/mL. After 48 h of incubation, the medium was aspirated, 40 uL MTT salt (2.5 µg/mL) was added to each well and incubated for further four hours at 37 °C under 5% CO_2. Doxorubicin (DOX) was used as a positive control at 100 µg/mL, giving 100% lethality under the same conditions [30]. The absorbance was then measured at 595 nm and a reference wavelength of 620 nm. The inhibitory concentrations which cause inhibition of 50% (IC_{50}) and 90% (IC_{90}) of cells in 48 h were determined. The percentage of change in viability (%*P*) was calculated according to the following formula:

$$\%P = \left[\left(\frac{Reading\ of\ nanoparticles}{Reading\ of\ negative\ control} \right) - 1 \right] \times 100$$

2.4. Laboratory Manufacturing of Domiati Cheese

Fresh cow's milk (9 L), locally produced on the day of the experiment, was salted (10%) and pasteurized at 63 °C for 30 min. The prepared inoculum was added to the warmed milk (35–40 °C) in a count of 6 log CFU/mL. The inoculated milk was divided into three parts for further use as follows: part 1 is the control (contained *S. aureus* only), part 2 (contained yoghourt starter culture, a package of 100 g active cultured yoghourt manufactured by the Faculty of Agriculture, Assiut University, Egypt) and part 3 (contained 35 µg/mL NiO NPs, the MIC of nanoparticles). Then, Domiati cheese was manufactured according to Fahmi & Sharara [31]. Briefly, 10–15 mL/100 lb rennet (7 mL/1 L of milk) was added to the inoculated warm milk at 35–40 °C). Then, the recovered cheeses were stored in their whey in sterile glass containers at room temperature (25 °C and 80% relative humidity) (Figure 1).

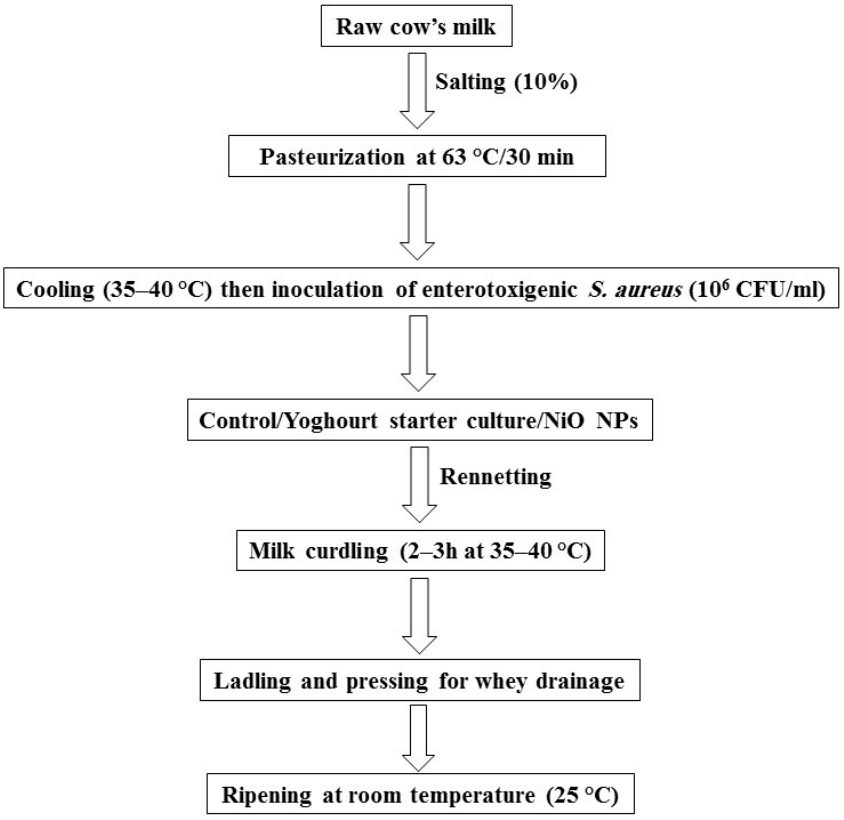

Figure 1. Flow diagram of Domiati cheese making. Control cheese; normal cheese without any treatment. Yoghourt starter culture cheese; cheese processed by addition of yoghourt. NiO NPs cheese; Domiati cheese inoculated with 35 µg/mL of NiO NPs.

2.5. Microbiological and Physico-Chemical Analysis of Domiati Cheese

Samples of the finished fresh cheeses were collected just after manufacturing and on a weekly basis during cheese storage and tested for *S. aureus* counts on the Baird-Parker agar (Oxoid Limited, Hampshire, UK), Aerobic Plate Count (APC) on plate count agar (Oxoid Limited, UK) and pH value using a pH meter (Manti Lab Solutions, Haryana, India). The moisture content was also measured using the method described by Atherton & Newlander [32] by heating the samples several times in a hot air oven. Total solids (%, *w*/*w*) were calculated by subtracting the obtained moisture % from 100%. Additionally, the NaCl concentration (% *w*/*w*) was measured in cheese samples according to Wilster et al. [33]. The NaCl concentration in the aqueous phase (or salt in water) was calculated as reported by Mehta [34] according to the following formula:

$$\text{NaCl in the aqueous phase [g/kg]} = (\text{Salt content in cheese [g/kg]}/\text{Water content of cheese [g/kg]}) \times 1000$$

2.6. Detection of Staphylococcal Enterotoxin A (SEA) Using ELISA Technique

Samples from the control and starter culture cheese were examined for the presence of SEA using an ELISA kit (RIDASCREENÒ SET A, B, C, D, E Art. No: R4101, R-Biopharm AG, Germany) according to Rahimi et al. [35]. Firstly, 10 g of thoroughly mashed cheese was mixed with 15 mL of phosphate buffer saline (PBS) and then vortexed for 15 min. The tubes were centrifuged for 10 min at 2500× *g* at room temperature and the supernatant was pipetted for further examination. In each microtiter strip, 100 µL of the prepared samples were added to wells. The strip was mixed gently and incubated for 60 min at room temperature. The wells were washed and 100 µL of the diluted enzyme conjugate was added. After incubation for 60 min and washing, 50 µL of the substrate and 50 µL of chromogen were added to each well. Then, 100 µL of stop solution was added after

incubation in a dark place. The absorbance was measured at 450 nm using an ELISA reader (Start Fax 2100, Westport, UK). The mean lower detection limit of the assay was set at 0.375 ng toxin/g.

2.7. Data Processing and Models' Development

The experimental data of APC (log CFU/mL or g) in the examined samples during manufacturing and ripening of Domiati cheese versus time (days) was collected in MS Excel (Microsoft Corporation) for each separated treatment. The lag time (LT, d), maximum growth rate (μ_{max}, log CFU/g) and maximum population density (MPD, log CFU/g) for each growth curve were calculated by fitting the observed data to the Baranyi model (Equation (1)) [36] by using the DMFit excel program (Institute of Food Research, Norwich, England). The performance and accuracy of the developed models were evaluated for the goodness-of-fit with the current data by measuring the Mean Squared Error (MSE) and coefficient of determination (R^2).

For the sake of the evaluation of the fate of *S. aureus* during storage against the addition of yoghourt starter culture and NiO NPs during processing and cheese ripening, two mathematical inactivation models were tested.

(i) The log-linear model was used as follows:

$$\log N(t) = \log N_0 - k_{\max} \cdot t \quad (1)$$

where *N(t)* is the number of survival cells (log CFU/g) at time *t*(d); N_0 corresponds to the initial inoculum level (log CFU/g); and k_{max} is defined as the maximum survival rate (log CFU/g).

(ii) The Weibull model was as follows:

$$\log_{10} N(t) = \log_{10} N_0 - \left(\frac{t}{\delta}\right)^p \quad (2)$$

where δ is the first-decimal reduction time (d) and p is a shape parameter.

Specifically, the Weibull-type model [37,38] has been previously used to describe the non-log-linear nature of survival curves thanks to its flexibility and simplicity. The freeware add-in GINaFit v1.6 [39] was used for the fitting procedure and statistical estimates.

2.8. Statistical Analysis

Experiments were done in triplicate to capture microbial variability. Descriptive statistics were calculated from the observed data with MS Excel (Microsoft Corporation). The statistical analysis performed consisted of mean comparison tests, univariate analysis of variance (ANOVA) followed by Tukey post-hoc test ($p < 0.05$) to evaluate significant differences in the concentration levels of APC and *S. aureus* as well as in the physico-chemical parameters in the dairy matrices. Moreover, to determine the relationship between the different concentrations of NiO NPs and the induced cell death in 48 h, ANOVA was used. The SPSS v22.0 software was used (Chicago, IL, USA).

3. Results

3.1. Assessment of Nickel Oxide Nanoparticles Cytotoxicity

In the present study, HECs were exposed to NiO NPs for 48 h and the cytotoxicity was measured by MTT assays. Results showed that cell viability was reduced and the degree of reduction was dose-dependent. In the MTT assay, the HEC viability was decreased to 95, 93, 74 and 59% for concentrations of 17.5 (0.5MIC), 35 (MIC), 70 (2MIC) and 140 (4 MIC) µg/mL, respectively (Figure 2).

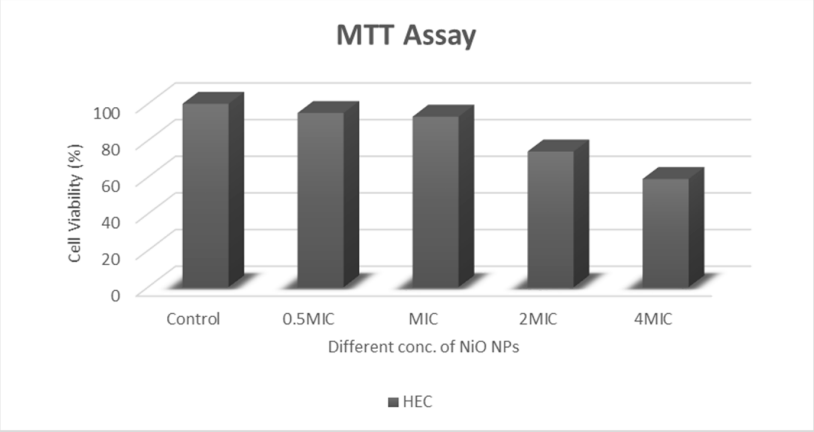

Figure 2. Cytotoxicity assessment of NiO NPs over normal human epithelial cell (HEC) line using the standard MTT.

3.2. Effect of Yoghourt Starter Culture and NiO NPs on S. aureus during Manufacture and Storage of Domiati Cheese

The average concentration of *S. aureus* in milk was 6.3 ± 0.02 log CFU/mL in the assayed treatments. After cheesemaking, the initial concentration of *S. aureus* remained at the same levels in the control samples (6.6 ± 1.02 log CFU/g) while a slight, non-significant decrease was found for cheese samples with the addition of yoghourt (5.8 ± 0.15 log CFU/g). The microbial kinetics of *S. aureus* and APC in control Domiati cheeses are shown in Figure 3. At time 0 (the finished product), APC counts increased gradually till the 21st day of cheese ripening (reaching, 9 log CFU/g). It was found that Domiati cheeses cannot support *S. aureus* growth since counts remained constant until the 21st day of storage and decreased from day 28th until reaching 3.5 log CFU/g at day 35th of storage. pH values decreased over time until reaching 4.0 at the end of storage (Figure 3). In the case of cheese containing yoghourt starter culture, the average APC counts showed a 0.5 log reduction during manufacturing until the cheese was obtained, then increased gradually to reach its maximum growth (9.9 ± 0.20 log CFU/g) at the end of cheese storage (Figure 4). On the 21st day of ripening, the level of *S. aureus* declined drastically to reach the final concentration of 2.5 ± 0.06 log CFU/g (pH; 3.8) at the end of the experiment (Figure 4).

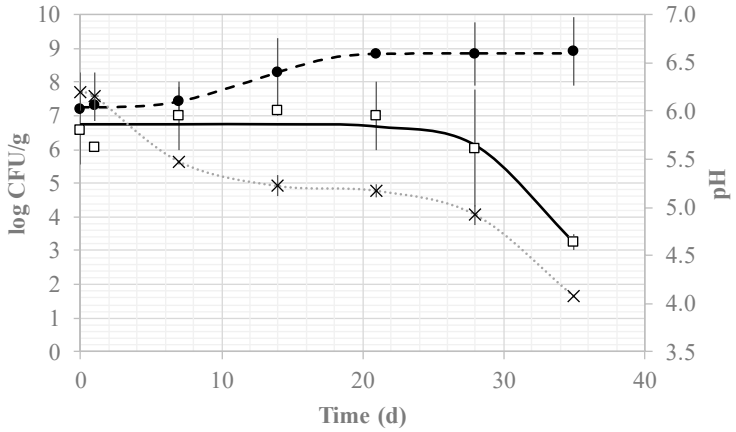

Figure 3. Total bacterial counts, *S. aureus* survival and pH evolution during Domiati cheese ripening for 35 d storage in control samples. • Observed growth of total bacterial counts (dashed line corresponds to the fitted Baranyi model); □ Observed survival of *S. aureus* (solid line corresponds to the fitted Weibull model); × pH evolution.

After examining the antibacterial effect of different concentrations of the studied nanoparticles, 35 µg/mL was the minimum inhibitory concentration (MIC) that could inhibit the enterotoxigenic *S. aureus* strains. Then, Domiati cheese was manufactured from

cow's milk formulated with 35 µg/mL NiO NPs and the periodical examination showed that there was a drastic decrease in the number of bacterial cells during the manufacture and ripening of cheeses with the added NiO NPs versus the control samples ($p < 0.05$). In other words, the count of *S. aureus* was reduced directly after manufacturing of NiO NPs Domiati cheese (Figure 5). In curd, the population of the microbe decreased by 0.5 log CFU/g, while one log reduction was observed in the finished product (zero time). Additionally, *S. aureus* rapidly decreased during cheese ripening until becoming undetectable on the 21st day of storage (Figure 5). For APC, there was a continuous increase in the total bacterial count from 6. 8 log CFU/g that reached 8.5 log CFU/g during the manufacturing and ripening of this kind of cheese with a clear decrease in pH (from 6.5 to 3.65) (Figure 5).

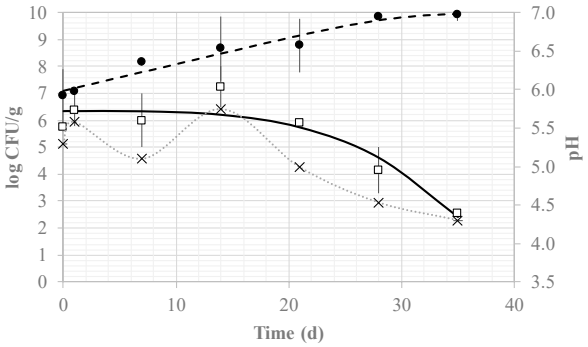

Figure 4. Total bacterial counts, *S. aureus* survival and pH evolution during Domiati cheese ripening for 35d storage after the addition of a yoghourt starter culture. • Observed growth of total bacterial counts (dashed line corresponds to the fitted Baranyi model); □ Observed survival of *S. aureus* (solid line corresponds to the fitted Weibull model); × pH evolution.

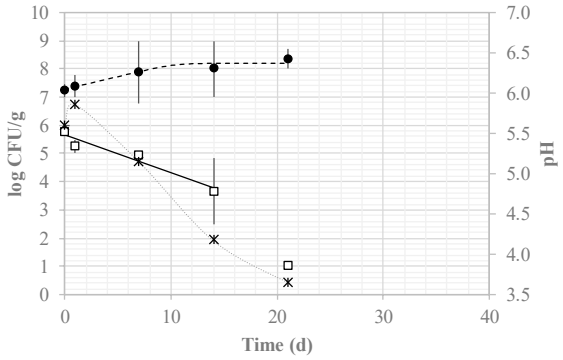

Figure 5. Total bacterial counts, *S. aureus* survival and pH evolution during Domiati cheese ripening for 35d storage after the addition of nickel oxide nanoparticles (NiO NPs). • Observed growth of total bacterial counts (dashed line corresponds to the fitted Baranyi model); □ Observed survival of *S. aureus* (solid line corresponds to the fitted Weibull model); × pH evolution.

Altogether, the bacterial load in the examined cheese samples was variable between groups. The initial concentration of *S. aureus* in milk did not change in the control group (6.6 ± 1.02 log CFU/g) after cheese making. A slight decrease in *S. aureus* count was observed in cheese containing yoghourt starter culture and NiO NPs (5.8 ± 0.15 log CFU/g & 5.3 log CFU/g, respectively) at time 0. During cheese ripening, the level of *S. aureus* started to decrease on days 28th and 21st in the control and cheese containing the yoghourt starter culture, respectively. Further, the concentration of the organism decreased gradually to 3.2 log CFU/g and 2.5 log CFU/g at the end of the experiment (5 weeks) in such groups, respectively. On the other hand, NiO NPs prevent the increase of *S. aureus* from the first step of manufacture (5.3 log CFU/g) with undetectable levels on the 21st day of cheese ripening. Moreover, there was a significant reduction ($p < 0.05$) in the mean count of *S. aureus* strains

in presence of NiO NPs in comparison to the control during the manufacturing and ripening of Domiati cheese (Figure 6).

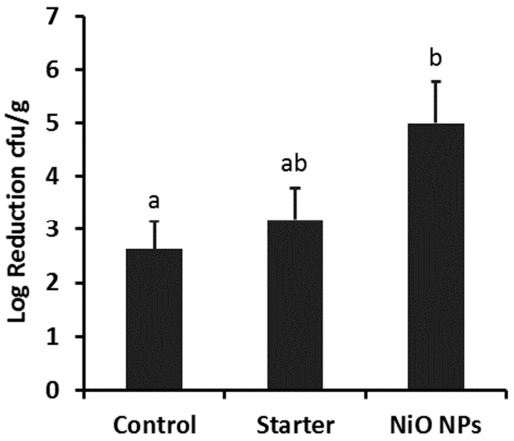

Figure 6. Comparison between the inactivation levels of yoghurt starter culture and nickel oxide nanoparticles on the count of *S. aureus* versus control during manufacture and storage of Domiati cheese. Letters a and b describe significant differences at 95% confidence level according to the Tukey post-hoc test.

In a modeling approach, the data of APC from the current study were further analyzed using the Baranyi model. For the fitted treatments, there was good agreement between predicted and observed values as seen in Table 1 (R^2 = 0.996, 0.934 and 0.850 for control, yoghourt starter culture and NiO NPs cheese samples, respectively). No significant differences were obtained between the estimated μ_{max} ($p \geq 0.05$), though it was slightly higher in control samples than in cheeses with yoghourt starter culture or NiO NPs (Table 1). The Weibull inactivation model was used to examine the survival of *S. aureus* in control and yoghourt starter culture Domiati cheese. Estimated parameters are presented in Table 2. In both cases, the fitted models reflected adequately the convexity due to the initial resistance of *S. aureus* population followed by a decay period (Figrues 3 and 4). Significant differences were obtained between the kinetic parameters of the Weibull models ($p < 0.05$) being the δ parameter (first-decimal reduction time) higher for control samples than for the cheeses with yoghourt starter culture (Table 2). While in NiO NPs cheese, a linear inactivation pattern was shown for *S. aureus* with a mean inactivation rate (k_{max}) of 0.3 log CFU/g (Table 2).

3.3. Evaluation of SEA Using RIDASCREENÒ SET A, B, C, D, E ELISA Kit

Samples of Domiati cheese from all studied groups (control, Yoghourt starter culture & NiO NPs) were analyzed for the detection of SEA using the ELISA technique. Of particular note, SEA was detected in control Domiati cheese and failed to be detected in presence of either yoghourt starter culture or NiO NPs.

3.4. Physico-CHEMICAL ANALYSIS of Domiati Cheese

Table 3 presents the chemical examination of Domiati cheese at all times of cheese manufacture and ripening. It can be concluded that there were no substantial changes in moisture and total solids constituents between cheese containing yoghourt starter culture or NiO NPs in comparison with the control ($p \geq 0.05$). In all categories of cheese, chemical examination verified that the pH and moisture content of the obtained cheeses decrease during the storage of Domiati cheese. On the contrary, the total solids % was increased from the initial storage time until the end of the study. Regarding NaCl % (in the aqueous phase), it increased gradually during ripening of all types of cheese, reaching its highest level in the cheese with a starter culture.

Table 1. Estimated growth parameters of Aerobic Plate Counts (APC) in Domiati cheeses using Baranyi model.

Treatments	Estimated Kinetic Parameters					
	LT [1] (d)	μ_{max} (log CFU/d) [2]	N_0 (log CFU/g) [3]	MPD (log CFU/g) [4]	SE of Fit	R^2
Control	6.343 ± 0.822	0.137 ± 0.015 ^{A}	7.247 ± 0.037 ^{A}	8.857 ± 0.031 ^{B}	0.052	0.996
Yoghourt starter culture	- [6]	0.099 ± 0.016 ^{A}	7.084 ± 0.199 ^{A}	9.979 ± 0.368 ^{C}	0.306	0.934
NiO NPs [5]	-	0.089 ± 0.034 ^{A}	7.269 ± 0.135 ^{A}	8.180 ± 0.127 ^{A}	0.176	0.850

[1] Lag time; [2] Maximum growth rate; [3] Initial concentration; [4] Maximum Population Density; [5] Nickel Oxide Nanoparticles; [6] Not available. Means with different capital superscripts denote significant differences (p-value < 0.05) between columns (p-value < 0.05).

Table 2. Estimated survival parameters of the enterotoxigenic *S. aureus* in all kinds of the examined cheeses using Weibull and linear models.

Treatments	Type of Model	Estimated Parameters									
		Δ	p	MSE	R^2	R^2 Adjusted	N_0 (log CFU/g)	k_{max} (log CFU/d)	MSE	R^2	R^2 Adjusted
Control	Weibull	29.71 ± 2.31 ^{A}	7.70 ± 3.51 ^{A}	0.20	0.92	0.89	-	-	0.19	0.93	0.90
Yoghurt starter culture	Weibull	24.05 ± 4.17 ^{B}	3.66 ± 1.66 ^{B}	0.44	0.88	0.82	-	-	0.38	0.89	0.84
NiO NPs [1]	Linear	- [2]	-	-	-	-	5.65 ± 0.19	0.31 ± 0.06	0.08	0.94	0.91

[1] Nickel Oxide Nanoparticles; [2] Not available; Means with different capital superscripts denote significant differences (p-value < 0.05) between columns (p-value < 0.05).

Table 3. Physico-chemical analysis (moisture, total solids & NaCl%) of control, yoghurt starter culture and nickel oxide nanoparticles cheeses during cheese ripening.

Time (d)	Control			Yoghourt Starter			NiO NPs [2]		
	Moisture%	TS% [1]	NaCl%	Moisture%	TS%	NaCl%	Moisture%	TS%	NaCl%
0	58.0 ± 2.8 ^{Aa}	42.0 ± 2.8 ^{Aa}	1.9 ± 0.1 ^{Ba}	59.0 ± 2.8 ^{Aa}	41.0 ± 1.4 ^{Aa}	2.4 ± 0.1 ^{Aa}	46.2 ± 1.7 ^{Ba}	35.8 ± 1.7 ^{Ba}	2.5 ± 0.0 ^{Aa}
7	55.3 ± 3.2 ^{Aa}	44.7 ± 3.2 ^{Aa}	2.4 ± 0.1 ^{Bb}	54.5 ± 0.0 ^{Ab}	45.5 ± 0.0 ^{Ab}	2.9 ± 0.1 ^{Ab}	59.8 ± 5.9 ^{Ab}	40.2 ± 5.9 ^{Bab}	3.0 ± 0.4 ^{ABab}
14	52.4 ± 2.8 ^{Aab}	47.6 ± 2.8 ^{Aa}	2.8 ± 0.1 ^{Ac}	52.8 ± 2.8 ^{Ab}	47.2 ± 2.8 ^{Ab}	3.0 ± 0.1 ^{Ab}	54.8 ± 2.6 ^{Ab}	45.2 ± 2.6 ^{Ab}	2.9 ± 0.6 ^{Aab}
21	51.5 ± 1.4 ^{Ab}	48.5 ± 1.4 ^{Aab}	2.9 ± 0.1 ^{Ac}	51.4 ± 1.6 ^{Ab}	48.6 ± 1.6 ^{Ab}	3.3 ± 0.3 ^{Ab}	50.9 ± 0.1 ^{Ab}	49.1 ± 0.1 ^{Ab}	3.2 ± 0.1 ^{Ab}
28	49.2 ± 0.0 ^{Ab}	50.8 ± 0.0 ^{Ab}	3.2 ± 0.1 ^{Ac}	45.8 ± 1.6 ^{Ac}	54.2 ± 1.6 ^{Bc}	3.9 ± 0.4 ^{Abc}	- [3]	-	-
35	44.7 ± 0.4 ^{Ac}	55.3 ± 0.4 ^{Ac}	3.4 ± 0.3 ^{Ac}	44.5 ± 3.1 ^{Ac}	55.5 ± 3.1 ^{Ac}	4.5 ± 0.3 ^{Bc}	-	-	-

[1] TS; Total Solids; [2] Nickel Oxide nanoparticles; [3] Not examined; Means with different capital superscripts denote significant differences (p-value < 0.05) between rows while those with different lowercase superscripts indicate significant differences between columns (p-value < 0.05).

4. Discussion

The current study elucidated the inhibitory effect of yoghourt starter culture and NiO NPs on *S. aureus* and enterotoxin production during the processing and ripening of Domiati cheese. Strikingly, adding NiO NPs to Domiati cheese could induce a higher antibacterial effect on *S. aureus* than yoghourt starter culture. Hence, NiO NPs could be a useful candidate against *S. aureus* and enterotoxin production in the dairy industry. Besides, using yoghourt starter culture during the processing of Domiati cheese could prevent enterotoxin production even in presence of a high level of contamination with the enterotoxigenic *S. aureus* in milk that will be used for cheese making.

The present study revealed that *S. aureus* decreased one week earlier in starter cultured cheese and in a rapid manner reaching a low level at the end of the experiment versus the control. This indicated that starter culture had some inhibitory effect on *S. aureus* during the ripening process of Domiati cheese. Notably, the addition of yoghourt could interfere with the production of staphylococcal enterotoxins (SEA). Hence, cheese manufactured with the addition of yoghourt does not create risk for consumers even if it is contaminated with high counts of the enterotoxigenic *S. aureus*. Figure 6 illustrated that the log reduction of the enterotoxigenic *S. aureus* in control and yoghourt starter culture Domiati cheese did not differ significantly. Although *S. aureus* grows over a wide range of pH between four to 10 with an optimum of six to seven, and can survive salt concentrations from 0 to 20% with an optimum of 0% [40]. Herein, the decline in the mean count of *S. aureus* may be due to the synergistic effect of low pH and high NaCl concentration that leads to shrinkage and death of the bacterial cells [41]. Similarly, Al-Nabulsi et al. [42] reported that the combination of a starter culture, high salt concentration (15%), low temperature, and pH (∼5.2) had inhibitory effects on the growth of *S. aureus* and may prevent the enterotoxin production during white-brined cheese making.

There are several studies, with different outcomes, evaluating the effect of starter culture, NaCl % and storage temperature on the development of *S. aureus* during the manufacture and storage of different types of cheeses. The current result was in accordance with Al-Nabulsi et al. [42] who examined the effect of 0.5% starter culture on *S. aureus* during the manufacture of white cheese stored in pasteurized brine with different salt concentrations. The author reported that the addition of starter cultures did not significantly change the total number of *S. aureus*, but only increase the number of the organisms in the examined cheese from the 15th to the 60th day of cheese ripening and then declined at day 90th of ripening. It is concluded from the study that the count of *S. aureus* in cheese is the same in the presence or absence of the starter culture. In contrast, some of the previous studies stated that the microbial load of *S. aureus* decreased during cheese ripening [6,14], but only three out of nine examined cheese samples showed a decrease in *S. aureus* counts. There are some differences between the obtained results in the present study and that reported before, this may be due to using different kinds of cheese, type and amount of starter culture, the level of *S. aureus*, NaCl % or the storage temperature. Overall, the outcome from the current study and some of the previous studies is that the starter culture had not a significant inhibitory effect on *S. aureus* versus to control. This indicated that the decrease in the development of *S. aureus* during cheese ripening is a normal mechanism of the pathogen to mimic the chemical and microbiological changes that occur during the ripening of cheese. Otherwise, the addition of yoghourt starter culture herein is recommended to prevent enterotoxin production and avoid food poisoning that may occur due to consumption of the contaminated cheese. However, detecting the amount of the produced SEA toxin in the examined cheese samples is a limitation in the existing study. A quantitative ELISA kit with a much lower detection limit than 0.375 ng toxin/g is required be used in the future. So, it will be easier and more accurate to investigate the effect of the different treatments.

On the other hand, small-sized NiO NPs (20 nm) were used in this study so that they can easily penetrate the bacterial cell membrane leading to a better antibacterial effect [43]. After examining the antibacterial effect of different concentrations of the studied

nanoparticles, 35 μg/mL was the minimum inhibitory concentration (MIC) that could inhibit the enterotoxigenic *S. aureus* strains. This result was lower than that obtained by Suresh et al. [28] who tested NiO NPs for their antibacterial activity against *S. aureus* and reported that the MIC of NiO NPs was 60 μg/mL. In contrast, a lower MIC value of NiO NPs (0.21 μg/mL milk) was investigated by Haghshenas & Faraji [26]. Herein, we examined the cytotoxicity of NiO NPs on HECs for 48 h using MTT assays. Our findings indicated that the MIC used in this study is consider safe on the viability of HECs (93%) and it does not have IC_{50} or IC_{90}. The obtained results are consistent with previous studies suggesting that the cytotoxicity of NiO NPs is mediated through ROS generation and oxidative stress [44,45]. Haritha et al. [46] reported that the cytotoxic effects of NiO NPs are studied in cultured human colorectal cancer cells (HCT-116), which have exhibited significant anticancer activity with 55 μg/mL at IC_{50}. Hence, NiO NPs may offer a safe potential for many diseases management and can be applied to different medical and industrial applications [16,46]. Importantly, the cytotoxic effect of NiO NPs on intestinal epithelial cells is not clear. To confirm the anti-toxic effect of such particles, we had to examine their cytotoxicity through oral administration of lab animals besides MTT assay. This limitation in the current study will be avoided and compensated for in future studies.

Domiati cheese that was manufactured from cow's milk formulated with 35 μg/mL NiO NPs showed a drastic decrease in the number of bacterial cells (*S. aureus*) during the processing and ripening of cheeses versus the control samples ($p < 0.05$). It is clear from the present study that NiO NPs had a favorable effect on the inhibition of enterotoxigenic *S. aureus* strains in cheese and can be used to avoid the contamination of such products with *S. aureus*. Furthermore, the addition of NiO NPs to cheese could prevent enterotoxins production from *S. aureus*. Similarly, Behera et al. [47] reported a strong antibacterial effect of NiO NPs against Gram-positive bacteria compared to Gram-negative bacteria. Importantly, the main effect of such particles may be due to the ability of NiO Nps to generate oxidative stress (ROS) that resulted in rapid membrane damage leading to bacterial cell death [47] beside the low pH and high NaCl concentration in Domiati cheese during ripening. Thus, these particles can be one of the most promising preventive measures that may be applied in dairy industry in the near future. However, there are some limitations in such part of the present study as the strong antibacterial mechanism of NiO NPs against *S. aureus* in Domiati cheese that is not fully clear. Hence, the mechanism needs to be addressed in future reasearch. Regarding the chemical examination of all types of cheeses, there were not any significant changes in the examined parameters in comparison to control. However, NaCl % (in aqueous phase) increased gradually during ripening of cheese and reaching its highest level in the cheese with yoghourt starter culture. This can be attributed to the low pH in such kind of cheese as reported by Geurts, Walstra & Mulder [48] who showed the decrease in the uptake of salt by the cheeses during brining when the pH values increased. Altogether, when comparing the antibacterial effect of yoghourt starter culture and NiO NPs on the enterotoxigenic *S. aureus* during Domiati cheese making, it was obvious that nanoparticles were more effective.

5. Conclusions

The current study investigated that the addition of NiO NPs to milk for manufacturing of Domiati cheese can be used as a preventive measure to inhibit *S. aureus* and prevent the enterotoxins production which is the predominant cause of staphylococcal food poisoning in dairy products. Additionally, using yoghourt in the processing of Domiati cheese could prevent the enterotoxin production even in the presence of a high level of contamination with the enterotoxigenic *S. aureus* in milk that will be used for the processing of cheese. This study could be useful for cheese makers and stakeholders to set specific formulations based on the use of novel preservation technologies to inhibit enterotoxigenic *S. aureus* growth in Domiati cheeses. The current study examined the antibacterial effect of NiO NPs on *S. aureus* and enterotoxin production for research only, not for direct application in the food industry.

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