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Challenge Test for Assessing the Growth Potential of Listeria Monocytogenes in Greek Soft Cheese (Anthotyros)

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Abstract: Foodborne listeriosis is one of the most serious and severe foodborne diseases, with a high mortality rate worldwide. *Listeria monocytogenes' (Lm)* ability to survive under a diversity of conditions makes it a threat for food safety. Soft and semisoft cheeses are common RTE foods that support survival and growth of *Lm* due to their high moisture content and favorable pH. The aim of the present study was to assess, after artificial inoculation, the growth potential (Δ) of *Lm* in vacuum packed RTE soft Greek Anthotyros cheese. Growth potential of *Lm* was determined according to the new version of "EURL *Lm* Technical Guidance Document on challenge tests and durability studies for assessing shelf life of ready-to-eat foods related to *Lm*". The results of our study revealed that the growth potential of *Lm* was (Δ) = 4.93 $\log_{10}$ CFU/g, indicating that the specific soft cheese "anthotyros" is a "Ready to eat food able to support growth of *Lm* and classified in food safety category 1.2 in the (EC) Regulation 2073/2005". That means that the food business operator (FBO) must comply with the criteria that define that the bacterium must not be detected in 25 g (n = 5, c = 0) at the time of leaving the production plant. Through this study, we try to highlight the need for FBO to conduct relevant research to ensure that the ready-to-eat products which they produce, during their shelf life, do not support the growth of *Lm*.

Keywords: *Listeria monocytogenes*; challenge test; growth potential; soft cheese; ready-to-eat food; food safety

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1. Introduction

Listeria monocytogenes (*Lm*) is a Gram-positive aerobic and optional anaerobic bacterium, that is ubiquitous in the environment, in water, soil, and feces. *Lm* is a psychotropic bacterium that grows even in cooling temperatures and has great resistance to the environment; to various stresses, such as sanitizers, refrigeration and acidic environments; and food processes procedures, e.g., smoking, freezing [1–3]. *Lm* was identified in the 1980s as a food-borne pathogen [4]. Once in the food-processing environment, it can survive for long periods of time even in hostile environment, partially due to its ability to form biofilms [5,6]. If measures are not taken to effectively control and monitor *Lm* in the food-processing environment, the bacterium may persist, creating a potential cross-contamination route to the food. *Lm* is responsible for an infection called listeriosis. Listeriosis is a relatively rare disease, rarely affecting healthy adults, but is life-threatening, mainly for immunocompromised individuals, the elderly, pregnant women, and infants, causing very serious adverse effects, such as abortion, meningitis, sepsis, stillbirth, nerve disease, and even death [7]. One of the main routes of transmission of *Lm* is through contaminated food. Various ready-to-eat foods (RTE) have been identified as potential carriers of *Lm*. Most cases are sporadic, but major outbreaks have been also reported to be associated with soft

cheese from pasteurized milk [8,9], smoked-salted fish [10], salads [11], pâte [12], meat and meat products, [13–16] and processed foods that have been refrigerated for some time [17].

In the European Union in 2020, listeriosis was the fifth most commonly reported zoonosis in humans with a notification rate of 0.42 cases per 100,000 population, presenting no statistically significant variation during the last years. The overall high EU case fatality (13.0%), makes listeriosis one of the most serious foodborne diseases under EU surveillance [18]. In Greece the notification rate of listeriosis is low. For the period 2004–2020, 0.11 cases per 100,000 population were reported; half of them were either immunocompromised, or pregnant or newborn. Among cases with a known outcome, the fatality rate was 23.6% [19].

European Regulation (EC) No. 2073/2005 [20] lays down the food safety criteria for certain microorganisms in food products. Criteria regarding the presence of *Lm* in RTE foods are strict, requiring the following: (i) in RTE products intended for infants and for special medical purposes *Lm* must not be detected in 25 g (n = 10, c = 0); (ii) in other RTE foods, different microbiological criteria are applied depending on the ability of the food product to support growth of *Lm*, identifying three conditions in which the growth of *Lm* is not permitted: pH $\leq$ 4.4, water activity (aw) $\leq$ 0.92, or a combination of pH $\leq$ 5.0 with aw $\leq$ 0.94 are considered sufficient to prevent growth of the pathogen. Thus, for RTE foods unable to support the growth of *Lm*, the levels of the bacterium should not exceed the limit of 100 CFU/g throughout the shelf life of the product. On the other hand, in RTE foods that are able to support the growth of *Lm*, the bacterium must not be detected in 25 g (n = 5, c = 0) at the time of leaving the production plant; if the producer can demonstrate that the product will not exceed the limit of 100 CFU/g throughout its shelf life, the level should be <100 CFU/g throughout the shelf life of the product.

European Regulation (EC) No. 2073/2005 [20] also stipulates that food safety is the Food Business Operators' (FBOs) responsibility. FBOs should conduct studies in order to evaluate the growth of *Lm* that may be present in the product during the shelf life, considering reasonably foreseen conditions/abuse during the processes of distribution, storage, and use of the food product. Growth assessment studies, so-called challenge tests, may be carried out in order to investigate the ability of appropriately inoculated microorganisms of concern to grow or survive in the product under different reasonably foreseeable storage conditions and thus, meet the regulation's requirement. FBOs, based on the assessment of results of a challenge test, [21–27] must adopt the most effective strategies within their production processes and for the subsequent storage of food during the marketing phase in order to produce safe products for consumers [28,29].

In 2021, the Commission has published the "EURL *Lm* Technical Guidance Document on challenge tests and durability studies for assessing shelf life of ready-to-eat foods related to *Lm*" [30]. Several studies have been conducted and published regarding challenge testing and monitoring of *Lm* growth in many different RTE foods [24,31]. As mentioned in a review paper by Gerard et al. [32], soft cheeses seem to pose a significant risk to consumers, because the pH and a_w of soft cheeses are favorable for the reproduction of *Lm.* In addition to listeriosis, *Lm* in soft cheeses possibly also leads to biogenic amine contamination via its acid stress reaction, and biogenic amine is generally regarded as a contaminant in cheeses [33]. In terms of temperature, it was observed that the proliferation of *Lm* is also slower at lower temperatures in soft and semisoft cheeses [34,35].

Cheese is a very popular food product in Greece, due to the high domestic production of dairy food and because of the associated health benefits and flavor. In 2022, Greece is among the five countries worldwide with the highest cheese consumption (51.5 pounds per capita per year) [36] and is the 19th country in rank of cheese production worldwide, producing 220,100 tons of cheese (20.40 kg per person) [37]. In 2020, Greece exported 602 million USD in cheese, making it the 16th largest exporter of cheese in the world. In the same year, cheese was the 6th most exported product in Greece [38]. Anthotyros, (literally translated as "flowery cheese"), is a traditional Greek fresh cheese. There are dry and fresh Anthotyros made with milk and whey from sheep or goats, sometimes in combination. It may be unpasteurized where law allows [39].

The aim of the present study was to assess, after artificial inoculation, the growth potential (Δ) of the pathogenic microorganism *Lm* in vacuum packed RTE soft Greek Anthotyros cheese, made from sheep's and goat's pasteurized milk, produced by a company in the Southwest of Greece, in order to determine whether or not it has the ability to support the growth of the pathogen.

2. Materials and Methods

2.1. Food Product-Storage Temperatures

The studied product was vacuum packed Anthotyros soft cheese, made from sheep's and goat's pasteurized milk without the addition of preservatives. The products were obtained from the producing company in the Southwest of Greece 2 days after production. The net weight of each sample is 250 g and the shelf life indicated on the package is 25 days. The product is distributed throughout Greece through retail sales and in a specific supermarket. Before starting the challenge test, information was provided from the producer about the duration and storage conditions of the product at all stages of the cold chain: production, transportation, retail, and consumer level. The storage temperature that was applied at the consumer level was based at a study about temperatures of the food cold chain at the consumer level in Europe, reported by Bonanno and Bergis [40]. The temperature conditions that were adapted in our study were: (i) 5 °C to simulate storage conditions at the producer and transportation level; (ii) 7 °C to simulate storage conditions at the retail level; and (iii) 10 °C to simulate storage conditions at the consumer level. We determined as "Day 0" the day of inoculation of the Anthotyros cheese with *Lm*, and "Day End" the end of the product's shelf life.

In order to estimate inter-batch variability, three different batches were studied (Batch 1, Batch 2, and Batch 3), from three different production days, consisting of 22 samples each (17 samples for the challenge test and 5 spare samples). The samples were transferred to the laboratory in thermoboxes with ice packs.

The Challenge test—the growth potential study was performed following, in detail, the last version of "EURL *Lm* Technical Guidance Document on challenge tests and durability studies for assessing shelf life of ready-to-eat foods related to *Lm*" [30] and ISO 20976-1:2019 [41].

2.2. Inoculum

The inoculation of the test units with *Lm* was performed upon receipt at the laboratory of the Anthotyros cheese samples (Day 0). In order to avoid bias associated with the use of a unique strain of *Lm*, a mix of three different strains isolated from different dairy products was used to inoculate cheeses.

Strain reference: 09CEB411*LM*, molecular serotype IIa, clonal complex 26, Sequence typing 26, isolated from cheese.

Strain reference: 17SEL82*LM*, molecular serotype IVb, clonal complex 6, Sequence typing 6, isolated from cheese.

Strain reference: 17SEL22*LM*, molecular serotype IIa, clonal complex 14, Sequence typing 91, isolated from (environment) milk production filter.

Before receiving each batch, Subculture 1 and Subculture 2 of each of the above strains were prepared according to the (EURL *Lm* TGD) [30]. The inoculum suspension was then prepared, consisting of equal concentration of liquid cultures of the three selected strains. *Lm* concentration of the inoculum was enumerated according to ISO 11290-2 [42]. The suspension was subsequently diluted in physiological water to obtain a concentration of 150 CFU/g in the product, the targeted contamination level. This level of contamination reduces the effect of measurement uncertainty associated with low numbers and it is also close to the Regulation 2073/2005 [20] food safety limit. In order to be able to inoculate the cheese with a total of all three *Lm* strains concentration of about 150 CFU/g, as EURL *Lm* TGD proposes [30], the inoculum preparation was standardized with many preliminary tests. The inoculum concentration of *Lm* for Batch 1 was: 1.4×10^4 CFU/mL, for Batch 2: 1.7×10^4 CFU/mL, and for Batch 3: 1.5×10^4 CFU/mL.

2.3. Inoculation-Contamination

In order to inoculate the test units and the control units, we unpacked the cheese units, inoculated them, and then repacked under vacuum (C200, MULTIVAC Sepp Haggenmüller GmbH & Co. KG., Wolfertschwenden, Germany). Following the EURL *Lm* TGD, the volume of the inoculum per cheese unit should not exceed 1% of the mass of the test unit. Ten points of inoculation were identified and each test unit was inoculated in-depth in 10 spots (about 0.2 mL per spot) using sterile syringes.

Additional units, called "control units" (n = 4) (blanks), injected with 0.9% NaCl in the same volume as *Lm* inoculum, kept under the same conditions as the test units, were used to determine the pH, a_w, and %NaCl, as well as the "background" microbial flora harbored in the food at "Day 0" and "Day End". Control units are used in order to evaluate any influences due to a change in the actual composition of the food which, in practice, results in the "same" physico-chemical conditions of inoculated test units [29]. One more control unit (n = 1) was used for the measurement of the storage temperatures of the test units. A thermal data logger (Elitech RC-5) in a dedicated control test unit was placed in the same incubator, as close as possible to the remaining test units, and the temperature values were recorded throughout the test. "Food Control Samples" (n = 5), not subjected to any preparation in order to verify the representativeness of the production, were also tested at "Day 0" for pH, a_w, and %NaCl, as well as the "background" microbial flora harbored in the product and for *Lm* detection (Table 1).

Table 1. Total number of units used for the challenge test assessing the growth potential in Anthotyros cheese, "EURL *Lm* TGD" [30].

Type of Units	Type of Analysis	Number of Units and Date of Analyze per Batch	
Test units	Enumeration of *Lm*	**7**	3 test units at "Day 0" and 1 test unit at 3 intermediate dates and 1 at "Day End"
Food control samples	Detection of *Lm*	**5**	5 at "Day 0"
	Measurement of physico-chemical characteristics		
	Enumeration of the associated microflora		
Control units	Measurement of physico-chemical characteristics	**4**	2 at "Day 0" and 2 at "Day End"
	Enumeration of the associated microflora		
	Temperature control	**1**	all along the test
Total number of units		**17**	

Seven samples (test units) at "Day 0" were inoculated with the inoculum of *Lm*. To ensure homogeneous contamination within the product, the packs were shaken manually for 1 min.

2.4. Experimental Design

Three of the 7 inoculated samples (test units) were analyzed for *Lm* enumeration at "Day 0" and the rest of the inoculated test units were placed in the 5 °C incubator for 2 days (to mimic storage conditions at the production and transport level). In order to estimate the intra-batch variability, 5 food control samples per batch at "Day 0" (the day that we received the samples and started our study) and two control units were tested for pH, a_w, % NaCl, and % fat content, as well as the "background" microbial flora—mesophilic aerobic count and lactic acid bacteria—harbored in the product and only the five food control samples for *Lm* detection.

After 2 days of incubation ("Day 2") at 5 °C, *Lm* was enumerated in one cheese test unit, and the remaining samples were further incubated at 7 °C for 5 days (to mimic storage

conditions at the retail level). After 7 days from "Day 0" ("Day 7"), *Lm* was enumerated in one cheese test unit, and the remaining samples continued to be incubated at 7 °C for 7 more days (to mimic storage conditions at the retail level), until "Day 14", when *Lm* was enumerated in one cheese test unit. The remaining samples were incubated at 10 °C to mimic storage conditions at the consumer level. At "Day 23", the last day of shelf life, "Day End" (the day of expiration of the product), *Lm* was enumerated in the last cheese test unit. Two control units were also analyzed for total microflora—mesophilic aerobic count and lactic acid bacteria—and for pH, a_w, and %NaCl. ISO standard methods were applied for all microbiological criteria testing [43–45].

2.5. Physico-Chemical Analyses

The Physico-Chemical analyses that were performed on food control samples and on control units at "Day 0" were: measurement of pH, a_w, % NaCl content, and % fat content. The physico-chemical analyses that were performed on control units at "Day End" were: measurement of pH, a_w, and % NaCl. Fat, salt in the aqueous phase, a_w, and pH values were determined according to AOAC procedures [46].

2.6. Data Analysis

For each batch, the growth potential (Δ) was calculated according to the formula: $\Delta = \log_{max} - \log I$, where $\log_{max}$ is the highest value of the *Lm* enumeration obtained from, at least, the 4 sampling points (excluding the sampling at "Day 0"), when one test unit is analysed per sampling point. The growth potential retained amongs all tested batches is the highest obtained Δ value [30]. If (Δ) is lower or equal to the limit of 0.5 $\log_{10}$, then it is assumed that the food is not able to support the growth of *Lm* (Category 1.3 of Regulation (EC) No 2073/2005). If (Δ) is higher than the limit of 0.5 $\log_{10}$, then it is assumed that the food is able to support the growth of *Lm* (Category 1.2 of Regulation (EC) No 2073/2005) [30].

The microbial growth was modelled using the Baranyi Growth Model [39]. For curve fitting, the program DMFit (IFR, Institute of Food Research, Reading, UK) was used (available at http://www.combase.cc/index.php/en/, accessed on 27 November 2022). Kinetic parameters of microbial growth, i.e., rate (k), lag phase (λ), and the maximum population (N_{max}) were estimated.

3. Results

3.1. Food Control Samples

Upon receipt of the samples at the lab, the temperature of Batch 1 was T = 3.2 °C, of Batch 2, T = 2.2 °C, and of Batch 3, T = 1.9 °C. The absence of *Lm* in the five food control samples at "Day 0" in each of the three batches was confirmed: "*Lm* was not detected in 25 g". Physico-chemical characteristics of the food control samples (n = 5 for each batch) indicate more or less stable physico-chemical conditions in the three different batches of the three different production dates, as described in Table 2.

Table 2. Physico-chemical characteristics of the five food control samples of each batch at "Day 0".

	Food Control Samples Batch 1				Food Control Samples Batch 2				Food Control Samples Batch 3			
	pH	a_w	% NaCl	% Fat	pH	a_w	% NaCl	% Fat	pH	a_w	% NaCl	% Fat
	6.85	0.962	0.82	10.8	6.72	0.957	0.76	10.6	6.84	0.94	0.83	12.4
	6.54	0.957	0.73	12.5	6.49	0.946	0.69	11.4	6.72	0.943	0.7	11.9
	6.52	0.956	-	-	6.54	0.973	-	-	6.47	0.952	-	-
	6.71	0.975	-	-	6.86	0.941	-	-	6.51	0.952	-	-
	6.63	0.949	-	-	6.74	0.957	-	-	6.53	0.978	-	-
Average	6.65	0.96	0.78	11.65	6.67	0.95	0.73	11.00	6.61	0.95	0.77	12.15
SD	0.14	0.01	0.06	1.20	0.15	0.01	0.05	0.57	0.16	0.01	0.09	0.35

3.2. Growth Potential of Listeria Monocytogenes

The behavior of *Lm* (in $\log_{10}$ CFU/g) in the three contaminated batches of ready-to-eat food (cheese) during storage at 5 °C, 7 °C, and 10 °C from the beginning of our research, "Day 0", to the end of shelf life, "Day End", is depicted in Table 3 and Figure 1. A significant increase in *Lm* was observed in all the tested batches in the inoculated samples (challenge test). No *Lm* was detected in non-inoculated cheese. *Lm* growth rate ranged 0.517–0.722 d^{-1}. No lag phase was observed in any of the tested batches. The maximum *Lm* population N_{max} ranged 6.4–7.1 log CFU/g at the end of storage period.

Table 3. *Lm* concentration ($\log_{10}$ CFU/g) in the 3 contaminated batches of ready-to-eat food (cheese) during storage at 5 °C, 7 °C, and 10 °C in the studied period ("Day 0" = day of inoculation, "Day End" = end of shelf life, that is, the 23rd day upon receipt of the batches at the 2nd day of production. The shelf life of the product was 25 days).

	***Lm* ($\log_{10}$ CFU/g)**			***Lm* % Percent Change**		
Day (Storage Temperature)	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
0	3.10	2.93	2.23			
2 (5 °C)	3.62	4.18	3.21	16.64	42.43	43.94
7 (7 °C)	6.43	7.07	6.92	77.47	69.28	115.24
14 (7 °C)	6.35	6.36	7.17	−1.15	−10.08	3.58
23 (10 °C)	6.36	6.71	7.04	0.08	5.63	−1.78

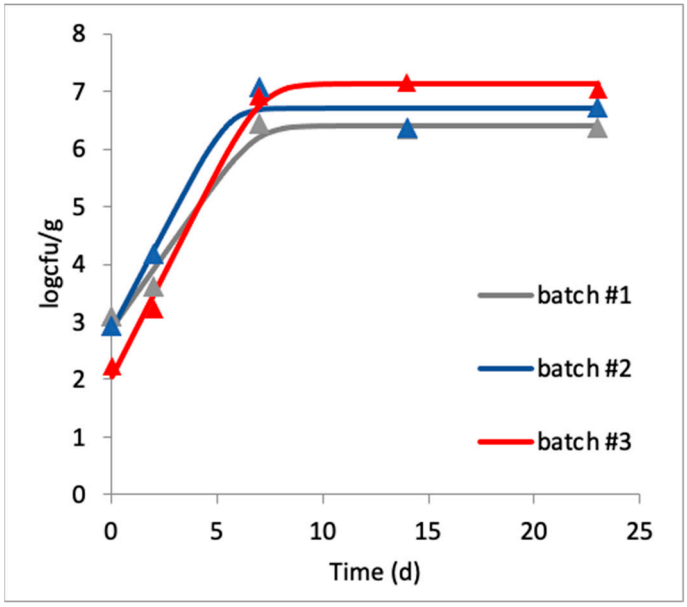

Figure 1. *Lm* concentration ($\log_{10}$ cfuCFU/g) in the 3 contaminated batches of ready-to-eat food (cheese) during storage at 5 °C, 7 °C, and 10 °C in the studied period ("Day 0" = day of inoculation, "Day End" = end of shelf life, that is, the 23rd day upon receipt of the batches at their 2nd day of production. The shelf life of the product was 25 days). (The dots indicate experimental values and the lines correspond to the predictions by the Baranyi model, R^2 = 0.956–0.987).

The growth potential (Δ), that is, the difference between the highest observed *Lm* concentration in $\log_{10}$ CFU/g during the challenge test ($\log_{max}$) and the initial concentration of *Lm* in $\log_{10}$ CFU/g ($\log_i$) at the beginning of the test, is presented in Table 4.

Table 4. Determination of growth potential (Δ).

Growth Potential (Δ) *Lm* $\log_{10}$ CFU/g			
	Batch 1	**Batch 2**	**Batch 3**
Day 0	3.15	3.02	2.20
	3.21	2.93	2.20
	2.94	2.82	2.29
Day 2	3.62	4.18	3.21
Day 7	6.43	7.07	6.92
Day 14	6.35	6.36	7.17
Day 23	6.36	6.71	7.04
The growth potential of *Lm* for each batch (Δ)Batch = $\log_{max} - \log_i$ (initial, at Day 0) in $\log_{10}$ CFU/g	Mean at Day 0 = 3.10 ±SD = 0.14 Δ = 6.43 − 3.10 = 3.33	Mean at Day 0 = 2.93 ±SD = 0.10 Δ = 7.07 − 2.93 = 4.14	Mean at Day 0 = 2.23 ±SD = 0.05 Δ = 7.17 − 2.23 = 4.94
Growth potential (Δ)	(Δ) = 4.94	Δ > 0.5 $\log_{10}$ CFU/g Anthotyros is a "Ready to eat food" able to support the growth of Lm" and is classified in food category 1.2 in the (EC) Regulation 2073/2005.	

For Batch 1, the Mean at Day 0 = 3.10, ±SD = 0.14 Δ = 6.43 − 3.10 = 3.33
For Batch 2, the Mean at Day 0 = 2.93, ±SD = 0.10 Δ = 7.07 − 2.93 = 4.14
For Batch 3, the Mean at Day 0 = 2.23, ±SD = 0.05 Δ = 7.17 −2.23 = 4.94

The growth potential (Δ) of "Anthotyros cheese" was 4.94. The calculated growth potential (Δ) was above the criterion 0.5 $\log_{10}$ CFU/g. Therefore, the studied Anthotyros is a "ready-to-eat" food able to support growth of *Lm* and classified in food safety category 1.2 in the (EC) Regulation 2073/2005.

3.3. Physico-Chemical Analysis of Control Units

Physico-chemical tests were performed on each batch, using two control units at "Day 0" and two control units at "Day End". The pH was, on average, at values around 6.5, however there was a significant decrease for all the examined batches from "Day 0" to "Day End" (Table 5), from 2.62% in Batch 1 to 5.96% in Batch 2. The determination of a_w had almost constant results until the end of the challenge test, with values between 0.95 and 0.96 (Table 5). Salinity values presented a swinging trend among the three batches. % NaCl increased from "Day 0" to "Day End" in two batches and decreased in Batch 2 (Table 5). pH and a_w values at the end of the Anthotyros cheese shelf life are still favorable for the multiplication of *Lm* if present just after production.

Table 5. Physico-chemical characteristics of the Anthotyros cheese (control units), at "Day 0" and at the end of shelf life, "Day-End".

Batch 1 Control Units					**Batch 2 Control Units**				**Batch 3 Control Units**			
Day 0	**pH**	**aw**	**% NaCl**	**% Fat**	**pH**	**aw**	**% NaCl**	**% Fat**	**pH**	**aw**	**% NaCl**	**% Fat**
1	6.52	0.974	0.75	13.4	6.75	0.96	0.88	12.60	6.59	0.98	0.72	10.50
2	6.47	0.943	0.67	11.6	6.85	0.97	0.73	12.80	6.72	0.96	0.66	11.90
Average	**6.50**	**0.96**	**0.71**	**12.50**	**6.80**	**0.97**	**0.81**	**12.70**	**6.66**	**0.97**	**0.69**	**11.20**
±SD	**0.04**	**0.94**	**0.67**	**11.60**	**0.07**	**0.01**	**0.11**	**0.14**	**0.09**	**0.01**	**0.04**	**0.99**
Day 23	**pH**	**aw**	**% NaCl**	**% fat**	**pH**	**aw**	**% NaCl**	**% fat**	**pH**	**aw**	**% NaCl**	**% fat**
1	6.21	0.95	0.69	NE *	6.30	0.97	0.72	NE *	6.34	0.96	0.78	NE *
2	6.44	0.95	0.83	NE *	6.49	0.95	0.67	NE *	6.29	0.97	0.72	NE *
Average	**6.33**	**0.95**	**0.76**		**6.40**	**0.96**	**0.70**		**6.32**	**0.96**	**0.75**	
±SD	**0.16**	**0.00**	**0.10**		**0.13**	**0.01**	**0.04**		**0.04**	**0.01**	**0.04**	

* Not Examined.

3.4. Microbiological Analysis

Background microbial flora harbored in the product at "Day 0" seems to increase for both microbiological criteria tested, mesophilic aerobic count and lactic acid bacteria, until "Day End". The concentration increase of mesophilic aerobic count and of lactic acid bacteria ranged from 39.09% for Batch 1 to 54.96% for Batch 3, and from 42.93% for Batch 2 to 48.44% for Batch 1, respectively (Table 6).

Table 6. Concentration of mesophilic aerobic count and lactic acid bacteria at "Day 0" and "Day End" in the control units of the three batches, and percentage of the increase of mesophilic aerobic count and lactic acid bacteria concentration during the studied period.

	Mesophilic Aerobic Count (log_{10} CFU/g)			Lactic Acid Bacteria (log_{10} CFU/g)		
Day	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
0 (5 °C)	6.08	5.51	5.52	5.53	5.40	5.41
23 (10 °C)	8.46	8.15	8.56	8.22	7.72	7.77
% concentration increase from Day 0 to "Day End"	39.09	47.79	54.96	48.44	42.93	43.56

4. Discussion

Foodborne listeriosis is one of the most serious and severe foodborne diseases, caused by the bacterium *Lm*. Listeriosis is a relatively rare disease with 0.1 to 10 cases per 1 million people per year (depending on the countries and regions of the world); however, the high mortality rate, as high as 30%, associated with this infection makes it a significant public health risk [47]. *Lm's* ability to survive under a diversity of conditions (psychotropic and halotolerant bacterium, facultative anaerobe, survives at temperatures a few degrees under the freezing point, and tolerates a wide pH range [48–50]), makes *Lm* a threat for food safety. Several food products are related to foodborne transmission of the pathogen. High risk foods include deli meat and ready-to-eat meat products (such as cooked, cured, and/or fermented meats and sausages), soft cheeses, and cold smoked fishery products. *Lm* is widely distributed in the natural environment, it survives and multiplies at low temperatures usually found in refrigerators, and when contaminating, it persists in food processing facilities. Many studies have documented the role of the food processing environment as a source of *Lm* contamination of finished products [51,52]. Therefore, the control of the pathogen in food processing facilities is a challenge, because it can persist for years in these facilities.

Lm poses a major problem in dairy facilities [53]. Many reported listeriosis outbreaks have been linked with dairy products [35,54]. Soft and semisoft cheeses are common RTE foods that support the survival and growth of *Lm* due to their high moisture content and favorable pH (4.6 to 7.5), representing a high risk of infection. Pasteurization inactivates *Lm* initially present in the raw milk, however pasteurized cheeses are still a major source of *Lm* infection due to post-pasteurization cross-contamination throughout the production and distribution chain [55].

As an RTE-food, *Lm* criteria in cheese has to comply with Regulation (EC) N°2073/2005, according to which food business operators should conduct studies in order to investigate the ability of the appropriately inoculated micro-organism of concern to grow or survive in the product under different reasonably foreseeable storage conditions.

According to the results of our study, the physico-chemical characteristics of the Greek soft cheese "Anthotyros" do support the growth of *Lm*, with pH $\geq$ 4.4 and water activity (a_w) $\geq$ 0.92. Even until the end of shelf life and with different storage conditions, pH and a_w were much higher than the limits that do not permit the growth of *Lm* (Table 5). Moreover, the natural acidification due to the progressive growth of "background" microbial flora during the storage of Anthotyros cheese did not cause a decline in pH level capable of

suppressing *Lm* growth. Additionally, the increased concentration of background microbial flora, through their antagonistic activity, did not manage to control *Lm* proliferation. As far as *Lm* growth potential is concerned, according to the results of our study, growth potential (Δ) = 4.94 $\log_{10}$ CFU/g, which indicates that the specific soft cheese "Anthotyros" is a "Ready to eat food able to support growth of *Lm* and classified in food safety category 1.2 in the (EC) Regulation 2073/2005". That means that the FBO must comply with the criteria that define that the bacterium must not be detected in 25 g (n = 5, c = 0) at the time of leaving the production plant.

Several similar studies have been conducted in order to assess the growth potential of *Lm* in different soft cheese products [24,35,56–58]. However, each cheese has its own characteristics, and two products with similar pH, a_w, dry matter, and microbial counts can lead to opposite behaviours of the pathogen [24].

The number of cases of listeriosis has increased worldwide during the last decade; in Greece, the notification rate of listeriosis is relatively low at 0.11 cases per 100,000 population for the period 2004–2020. Moreover, a survey conducted in 2011 in 137 various soft cheese products in the Greek retail market, in order to estimate the prevalence and contamination levels of *Lm*, found out that *Lm* was not isolated from any of the samples examined (0/137) [59]. Another study demonstrated that four out of ten different soft cheese products in a market in Greece were found to contain *Lm* [60]. Our study is expected to lead to the production of new results that will complement and enrich the knowledge of producers, traders, consumers, and researchers about the possibility of growth of the pathogenic microorganism *Lm*, and will help to improve production parameters and maintenance conditions, contributing to the protection of public health.

5. Conclusions

This is the first reported challenge test assessing the growth potential of *Lm* in the soft cheese "Anthotyros" in Greece according to the new version of "EURL *Lm* Technical Guidance Document on challenge tests and durability studies for assessing shelf life of ready-to-eat foods related to *Lm*, Version 4, 1 July 2021". This study was designed with the consideration of testing variable storage temperatures for the inoculated cheese with *Listeria monocytogenes*, in order to simulate the actual food supply chain.

The final aim of a challenge test is to assess the growth potential of artificially inoculated *Lm* in the food matrix with the maximum possible predictivity. Each food business operator must evaluate the results of each single challenge test and interpret them in the most correct way. Through this study, we aim to highlight the need for food business operators to conduct relevant research to ensure that the ready-to-eat products which they produce, during their shelf life, do not support the growth of *Listeria monocytogenes*. Conducting challenge tests on RTE products should be highly advised to FBOs as a valuable tool to be used in risk analysis activities, in order to verify the shelf life of their product based on food security and their product's own quality characteristics.

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