ISSN: 2458-8989

Natural and Engineering Sciences

NESciences, 2020, 5(2): 53-67

- RESEARCH ARTICLE-

The potential thermophilic Bacilli contaminants for dairy industry

Basar Karaca*, and Arzu Coleri Cihan

Ankara University, Faculty of Science, Department of Biology, Ankara, Turkey

Abstract

Though the biofilms of thermophilic, endospore-forming bacilli have received increasing interest in recent years, their biofilms are still unclear. In the current study, a comparative characterization of biofilm-forming patterns of totally 105 isolates and reference strains belonging to *Anoxybacillus* (85) and *Geobacillus* (20) genera were investigated. Various species belonging to these two genera have been screened for their biofilm production responses in milk, and their biofilm production patterns were also compared with their lactose and casein utilization. Thereby, this is the first report which displays strong biofilm producing behaviours of some thermophilic species in milk such as *G. thermoglucosidans*, *A. caldiproteolyticus*, *A. suryakundensis*, *A. salavatliensis*, and *A. kamchatkensis* subsp. *asaccharedens* except for well-known members like *G. steraothermophilis* and *A. flavithermus*. It was also assigned that elevated incubation temperature (65°C) had a stimulative effect on both biofilms forming capacities of *Geobacillus* and *Anoxybacillus*. Nevertheless, no direct correlation was found between biofilm formation capacity and lactose or casein catabolism characteristics. Finally, crystal violet binding assay, allowing the determination of the biofilm production amounts of microorganisms relatively in a short time, has been optimized. In this context, a methodological contribution has been provided to the literature in order to determine the possible biofilm production responses of thermophilic biofilms in the dairy industry.

Keywords:
Thermophilic bacilli, *Geobacillus*, *Anoxybacillus*, biofilm, dairy industry

Article history:
Received 25 October 2019, Accepted 07 April 2020, Available online 23 June 2020

Introduction

The term biofilm is defined as aggregates attached to a surface or interface, and microorganisms are embedded in polymeric components (EPS), which are produced and secreted into the extracellular environment. Biofilms can be detected on almost all abiotic or biotic surfaces such as human-made and natural water systems, food production environments, food packages, rocks,

* *Corresponding Author*: *Basar Karaca*, *e-mail*: *karaca@ankara.edu.tr*

glaciers, mucosal surfaces of host organisms, intravenous or urethral catheters and dental surfaces (Vert et al., 2012).

Thermophilic *Geobacillus* and *Anoxbacillus* members can grow at various temperatures ranging from 45°C to 75°C, widespread throughout the world, and can survive up to 110°C by endospore production. They can be isolated not only from many natural habitats but also from many human-made environments. High-temperature treatments in some industrial processes, including dairy industry, fruit juice pasteurization, canning, paper and sugar refining, gelatin, dried vegetables and fruit production, cause continuous contamination due to the accelerated thermophilic growth on behalf of their biofilm production and endospore formation (Denny, 1981; Hayes, 1985; De Clerck et al., 2004; Jay et al., 2005; Scott et al., 2007).

Geobacillus stearothermophilus and *Anoxybacillus flavithermus* are the most common species, isolated from dairy environments, and are defined as the primary source of continuous contamination for milk products, such as skim milk powder. Some product processing conditions in the dairy industry mainly contribute to the development of thermophilic bacilli biofilms. *G. stearothermophilus* and *A. flavithermus* can develop biofilms on the surfaces of milk powder processing units, such as heat exchange plates and evaporators, which may rise to high temperatures up to 70°C **(**Burgess et al., 2009; Hill & Smythe, 2012). Most of the bacilli detected in milk powders originate from the biofilm structures (Hill & Smythe, 2012). Above all, the endospore-forming ability of these bacilli makes them very difficult to eliminate. Therefore, the presence of these non-pathogenic bacilli is considered to be an indicator of products being processed under poor hygiene conditions. Also, the development of these bacilli in dairy products reduces food quality as a result of acid production and enzyme secretion. As the number of thermophilic bacilli in milk powder is essential for the determination of milk processing and market sales quality, the presence of thermophilic bacilli in high numbers also decreases the market sales in dairy products (Burgess et al., 2010; Hill & Smythe, 2012).

From a few decades, several trends such as the importance of ecological diversity, the evolutionary as well as the whole-genome sequencing, the metabolic diversity and the gene-content similarity have also become increasingly evident in addition to the previously used topics like relatedness for the biochemical and phenotypic traits for the new bacterial species delineation (Konstantinidis et al., 2006). A current whole-genome comparison study among the 29 members from genus *Geobacillus*, all of which were isolated from different thermal environments, with the other phylogenetically related taxa of *Anoxybacillus* and *Bacillus*, reflected their evolutionary history, their lifestyle and also their adaptation to a particular environment (Bezuidt et al., 2016). Bezuidt et al. (2016) also revealed that the habitat delineation with specific genomes linked to specific niches.

Based on the explanations above, in addition to the problems in current bacterial species identification, the ubiquitous of thermophilic bacilli in the worldwide, their different metabolic and physiologic properties, their thermophilic growth, endospore- and biofilm-forming capabilities, all make them the leading candidates of water or soil-borne spore associated contaminants for milk containing habitats dealing with dairy processing. Thermophilic members from genus *Geobacillus* and *Anoxybacillus* are currently represented with totally 42 bacterial species and six subspecies (http://www.bacterio.net/geobacillus.html, http://www.bacterio.net/anoxybacillus.html), and 28 of these species were used for the present screening assays. For this reason, the current study not only

depicts the biofilm responses of a wide diversity of species from *Anoxybacillus* and *Geobacillus* species in the whole milk by a comparative manner but also emphasizes on some new thermophilic species that may cause problems with their biofilm structures in the dairy industry, in addition to well known thermophilic species. Moreover, a crystal violet binding assay in a simply simulated dairy environment was also optimized in order to screen the biofilm-forming capabilities of various thermophilic species quickly.

Material and Methods

Bacterial cultures

Within the scope of the studies, 20 *Geobacillus* and 85 *Anoxybacillus* isolates and reference strains, were used from the culture collection of Ankara University, Biology Department, Microbiology Research Laboratory. The list of isolates and reference strains were as given in Table 1 (Cihan et al., 2011; Koc et al., 2015). All thermophilic bacilli, stored in glycerol stocks at -86°C, were first inoculated on MI (Medium I), starch; 10 g/L, peptone from casein; 5 g/L, yeast extract; 3 g/L, meat extract; 3 g/L, K_2HPO4; 3 g/L, KH_2PO4; 1g/L, agar 30 g/L, pH: 7.0 ± 0.2) agar for 18 h at 55°C (Suzuki et al., 1976). Following this step, the cultures were inoculated onto TSA (Tryptic Soy Agar) plates and then incubation carried out at 55°C for 18-24 h. Loopful of colonies were then inoculated into tubes containing 3 mL of TSB (Tryptic Soy Broth) to an OD600 nm of 0.2-0.4, and the incubation was carried out at 55°C and 170 rpm during 18 h. Finally, 200 µL of these active cultures were transferred into 5 mL TSB broth without NaCl, and the tubes were allowed to incubate for an additional 6 h at 55°C. These 6 h-cultures were used for all further experiments. This three-step inoculation process is necessary to accelerate the biofilm production capabilities of thermophilic bacilli by allowing them to remain in the vegetative cell form by delaying their transition to the sporulation phase (Kilic et al., 2017).

Table 1. List of isolates and reference strains used in this study. The origins of the isolates were presented in the previous studies of Cihan et al. (2011) and Koc et al. (2015) for *Geobacillus* and *Anoxybacillus* members, respectively.

Geobacillus (origins Cihan et al., 2011)	**Isolate and Reference Codes**
Geobacillus sp.	A353, C304, D413, E173b
G. kaustophilus	DSM 7263^T
G. stearothermophilus	DSM 22^T, ATCC 43223^T, DSM 5934^T, A113
G. thermodenitrificans	DSM 22625^T, DSM 465^T, D195
G. thermodenitrificans subsp. *calidus*	DSM 22628^T, DSM 22629^T
G. thermoglucosidans	DSM 2542^T, B84a
G. thermoleovorans	DSM 5366^T
G. toebii	E134, DSM 14590^T
G. vulcanii	DSM 13174^T
Anoxybacillus (origins Cihan et al., 2011; Koc et al., 2015)	
Anoxybacillus sp.	A321, A3210, E208a, E184aa, E184ab
A. amylolyticus	DSM 15939^T
A. ayderensis	NCIMB 13972
A. caldiproteolyticus	DSM 15730^T, A142, A146, A335, A392b, A394, A403, A404, A412b, A413, C226, D504, D621, D623, D494
A. calidus	DSM 25520^T
A. flavithermus	DSM 2641^T, A351a, A352b, A371, D486
A. flavithermus subsp. *flavithermus*	163

A. flavithermus-A. thermarum	D401a, D404, D463
A. flavithermus subp. *yunnanensis*	DSM 23293^T
A. gonensis	NCIMB 13933
A. kaynarcensis	DSM 21706^T
A. kestanbolensis	NCIB 13971
A. mongoliensis	DSM 19169^T
A. rupiensis	DSM 17127^T
A. suryakundensis	DSM 27374^T
A. tepidamans	DSM 16325^T
A. thermarum	DSM 17141^T
A. voinovskiensis	DSM 17075^T
A. kamchatkensis	DSM 14988^T, D433a
A. kamchatkensis subsp. *asaccharedens*	DSM 18375^T, D52b, D222b, D376b, E183, E184b, E206b, E208b, E272, E331, D371a, D394, D455, D594, E123, E237, E243, F81
A. salavatliensis	DSM 22626^T, A402b, A414, C163a, C245, D36, D98a, D202a, D203b, D204, D205, D211, D213, D214, D221a, D232a, D242b, D243 D392, D487, D503, D591, E206a

Determination of the amount of biofilm production in standard whole milk

The effects of standard whole milk on thermophilic biofilm formation was able to screened easily with the modified crystal violet binding methodology of Stepanović et al. (2000) by only changing the content of the medium used. The medium used in this study was prepared according to the recommendation of the manufacturer (Sigma-Aldrich, Darmstadt, Germany) and corresponds to the standard whole milk content (10% reconstituted skim milk). Inoculation preparation was performed as described above before biofilm sampling. After the inoculum preparation, 10 µL of the active culture for each thermophilic bacilli was transferred into the polystyrene microtiter plate wells containing 190 µL of standard whole milk. Two critical incubation temperatures (55°C and 65°C) were evaluated under static conditions up to 96-h incubation period. At the end of the incubation period, the planktonic counterparts were removed by rinsing the wells three times with physiological serum (0.85% NaCl) under aseptic conditions. The plates were fixed with 200 µL of 95% methanol for 15 min. Then, the plates were rinsed again, and 200 µL of 0.1% crystal violet solution was added to the wells. The plates were re-washed with distilled water to remove the unbound dye and dried after 30 min incubation. Finally, 200 µL of the ethanol: acetone (70:30 v/v) solution was added to each well for dissolving bound dye within the biofilm matrix. The optical density of the dissolved crystal violet dye was measured by Eliza reader at 595 nm (BioTek, USA). The amount of biofilm production was calculated by subtracting the averages of the negative control (containing only whole milk) wells from the mean of the absorbance values obtained from the test wells (whole milk and inoculum). Biofilm production capacities of thermophilic bacilli were categorized as non-producer ($OD \leq OD_{cut\ off}$), weak ($OD_{cut\ off} < OD \leq 2 \times OD_{cut\ off}$), moderate ($2 \times OD_{cut\ off} < OD \leq 4 \times OD_{cut\ off}$), and strong ($4 \times OD_{cut\ off} < OD$) based on the cut-off values obtained from negative control well absorbance values (Stepanović et al., 2000; Vestby et al., 2009). These modified steps were at least triplicated.

Determination of the lactose fermentation and casein hydrolysis

In lactose fermentation test, 0.5% of lactose was added to 5 mL of Basal medium (Claus and Berkeley, 1986). 0.5 mL active bacterial cultures were inoculated into the Basal medium containing lactose and Durham tube. The tubes were incubated for 48 h at 55°C under shaking conditions (170 rpm). At the end of the incubation time, the tubes were evaluated according to colour change and gas formation. The tubes with yellow colour were evaluated as positive for lactose fermentation. Two parallel test tubes were prepared for each bacterium. The test tubes without inocula were used as negative controls.

Standard whole milk supplied with 3% agar was also used to evaluate the presence of casein hydrolysis (Claus and Berkeley, 1986). After 24 h enrichment on TSA plates at 55°C, the loopful of these active cultures were inoculated by streak plate technique onto agar plates. The Petri dishes were incubated at 55°C for 48 h. The positive results were evaluated according to the presence of transparent zones formed by hydrolysis of casein on plates. All these experiments were at least duplicated.

Investigating the relationship between the biofilm formation and the lactose-casein utilization capabilities

The Chi-square test was performed to determine the possible correlations between the biofilm production responses and some biochemical characteristics (lactose fermentation/casein hydrolysing) of thermophilic bacilli.

Statistical analysis

Comparing the biofilm-forming amounts of thermophilic bacilli and temperature effects on biofilm-forming were evaluated by One-Way ANOVA and Paired T-Tests. The mean values of subgroups were compared according to Tukey's Test. The Chi-square test was also performed to illuminate the relationship exists on the categorical variables such as phenotypic and biofilm-forming characteristics. The bars of column charts were given as standard deviations (SPSS version 22.0, USA).

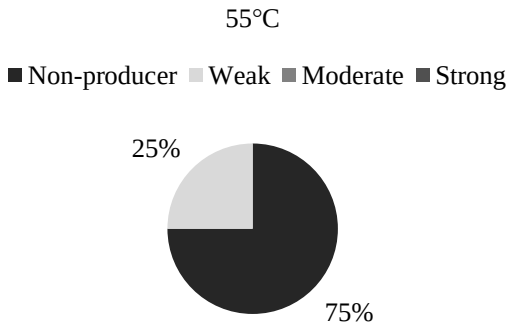
Results

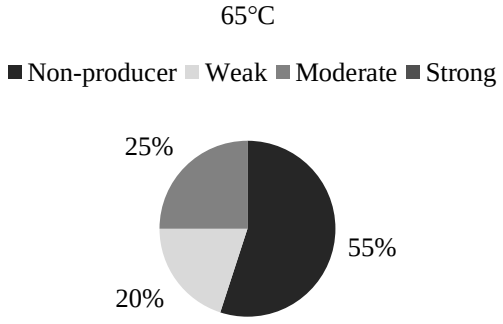
Biofilm production abilities of thermophilic bacilli in whole milk

The comparison of total biofilm yields at 55°C and 65°C incubation temperatures in *Geobacillus* members were found to be significantly different. Biofilm production yields and characteristics of all *Geobacillus* members on polystyrene surfaces containing skim milk were given in Figure 1 and Figure 2. The statistical analysis dealing with the biofilm production behaviours of species from genus *Geobacillus* at two different temperature values were also summarized in Table 2. Among *Geobacillus* species, 65°C was significantly superior to 55°C in terms of biofilm formation (Paired T-Test; $p<0.05$). No strong biofilm producers were detected in *Geobacillus* members in whole milk at both 55°C and 65°C. Fifteen of the 20 members and 11 of 20 were classified as non-producers at 55°C and 65°C, respectively. Only 5 out of the 20 *Geobacillus* members were identified as moderate biofilm producers at the elevated 65°C incubation temperature (Table 2).

Table 2. The correlation between biochemical characteristics (lactose fermentation-casein hydrolysing) and biofilm-forming capabilities in genus *Geobacillus*

	GEOBACILLUS				
	Biofilm forming characteristics				
55°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Lactose-fermenting	5	3	0	0	**8**
Non-lactose-fermenting	10	2	0	0	**12**
Total	**15**	**5**	**0**	**0**	**20**
*Chi-square test value: 1.111, *p=0.292*; The result is *not* significant at *p<0.05*.					
55°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Casein-hydrolysing	9	4	0	0	**13**
Non-casein-hydrolysing	6	1	0	0	**7**
Total	**15**	**5**	**0**	**0**	**20**
*Chi-square test value: 0.660, *p=0.417*; The result is *not* significant at *p<0.05*.					
	Biofilm forming characteristics				
65°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Lactose-fermenting	4	2	2	0	**8**
Non-lactose-fermenting	7	2	3	0	**12**
Total	**11**	**4**	**5**	**0**	**20**
*Chi-square test value: 0.228, *p=0.892*; The result is *not* significant at *p<0.05*.					
65°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Casein-hydrolysing	5	3	4	0	**12**
Non-casein-hydrolysing	6	1	1	0	**8**
Total	**11**	**4**	**5**	**0**	**20**
*Chi-square test value: 2.178, *p=0.337*; The result is *not* significant at *p<0.05*.					





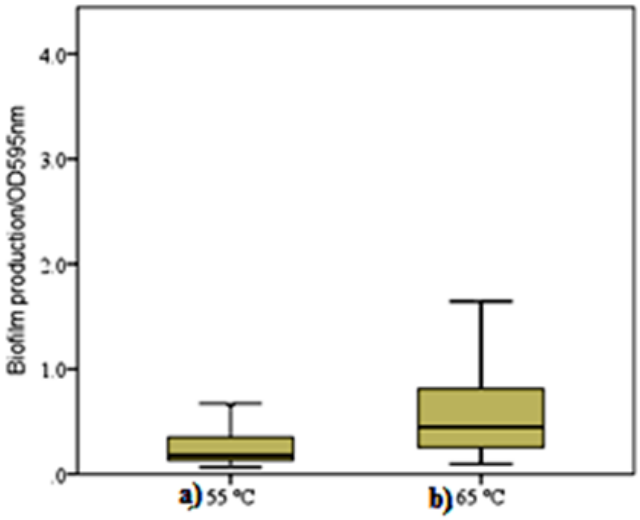


Figure 1. Total biofilm production yields of *Geobacillus* members at 55°C (a) and 65°C (b)

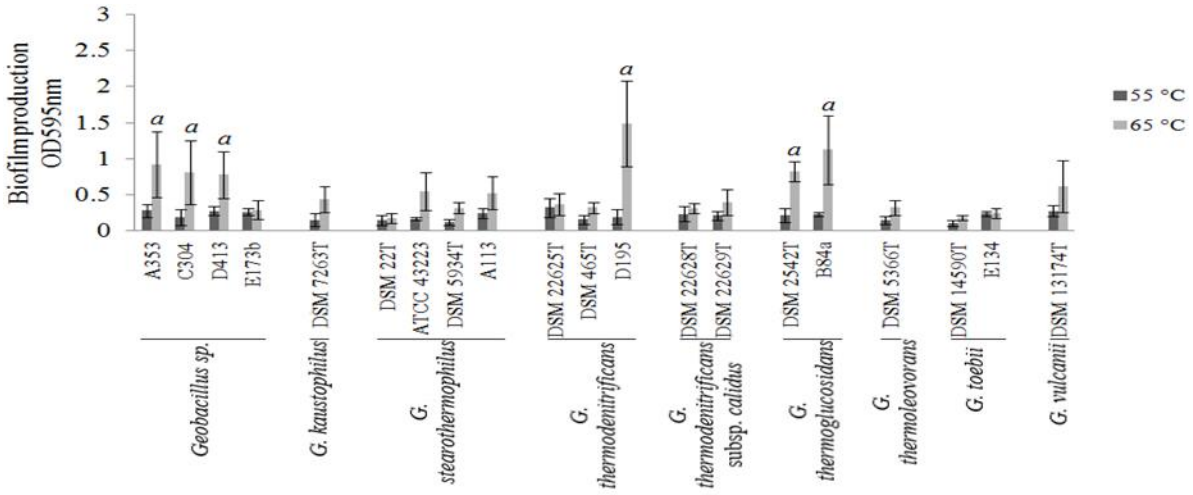


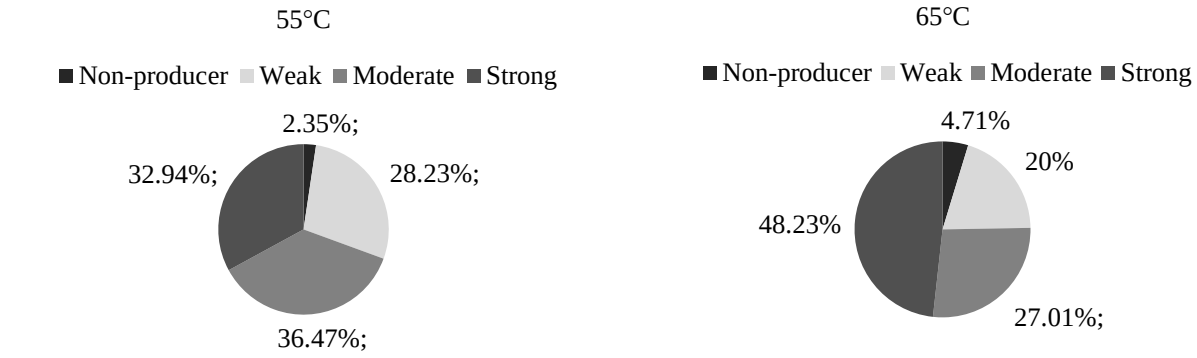
Figure 2. The amounts of biofilm production among *Geobacillus* members in milk at 55°C and 65°C.[a] indicates the significance in terms of biofilm formation and represent the best producers at 65°C.

Moreover, it was concluded that high incubation temperature promoted biofilm formation of *Geobacillus* members. The differences between biofilm formation values were found to be statistically significant at only 65°C (One-Way ANOVA; $p<0.05$). Generally, no difference was found at 55°C, but at 65°C; *Geobacillus* sp. A353, *Geobacillus* sp. C304, *Geobacillus* sp. D413, *G. thermodenitrificans* D195, *G. thermoglucosidans* B84a isolates and *G. thermoglucosidans* DSM 2542^T reference strain were all categorized as the strongest biofilm producers (Tukey's Test; $p<0.05$) among the genus *Geobacillus* and their biofilm production amounts were significantly effected from these two temperature values.

The distribution of biofilm production abilities among the species from genus *Anoxybacillus* in whole-milk content was also presented in Figure 3. The comparison of the total biofilm yields of *Anoxybacillus* members at 55°C and 65°C were found to be significantly different. 65°C was also much superior to 55°C as observed in the genus *Geobacillus* (Paired T-Test; $p<0.05$). It was suggested that *Anoxybacillus* members were prone to produce much more biofilm in contrast to *Geobacillus* members at both incubation temperatures. Twenty-eight members were categorized as strong producers at 55°C, whereas the numbers of bacilli from genus *Anoxybacillus*, producing strong biofilms, were 41 at 65°C (Table 3). Higher incubation temperature as 65°C was found to be more stimulative in terms of biofilm formation. The differences between biofilm formation values among the species belonging to genus *Anoxybacillus* were also found to be statistically significant at 55°C and 65°C as represented in Figure 4 (One-Way ANOVA; $p<0.05$).

Table 3. The correlation between biochemical characteristics (lactose fermentation-casein hydrolysing) and biofilm-forming capabilities in genus *Anoxybacillus*

	ANOXYBACILLUS				
	Biofilm forming characteristics				
55°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Lactose-fermenting	0	3	3	6	**12**
Non-lactose-fermenting	2	21	28	22	**73**
Total	**2**	**24**	**31**	**28**	**85**
***Chi-square test value: 3.572, *p=0.312*; The result is *not* significant at *p<0.05*.**					
55°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Casein-hydrolysing	2	10	6	9	**27**
Non-casein-hydrolysing	0	14	25	19	**58**
Total	**2**	**24**	**31**	**28**	**85**
***Chi-square test value: 3.617, *p=0.306*; The result is *not* significant at *p<0.05*.**					
	Biofilm forming characteristics				
65°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Lactose-fermenting	0	3	2	5	**10**
Non-lactose-fermenting	4	14	21	36	**75**
Total	**4**	**17**	**23**	**41**	**85**
***Chi-square test value: 1.239, *p=0.744*; The result is *not* significant at *p<0.05*.**					
65°C	**Non-producer**	**Weak**	**Moderate**	**Strong**	**Total**
Casein-hydrolysing	0	4	8	15	**27**
Non-casein-hydrolysing	4	13	15	26	**58**
Total	**4**	**17**	**23**	**41**	**85**
***Chi-square test value: 1.078, *p=0.782*; The result is *not* significant at *p<0.05*.**					



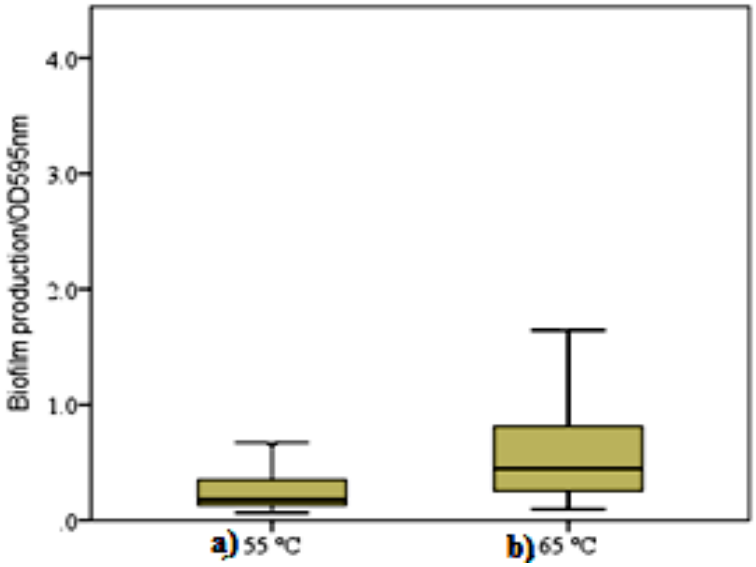


Figure 3. Total biofilm production yields of *Anoxybacillus* members at 55°C (a) and 65°C (b)

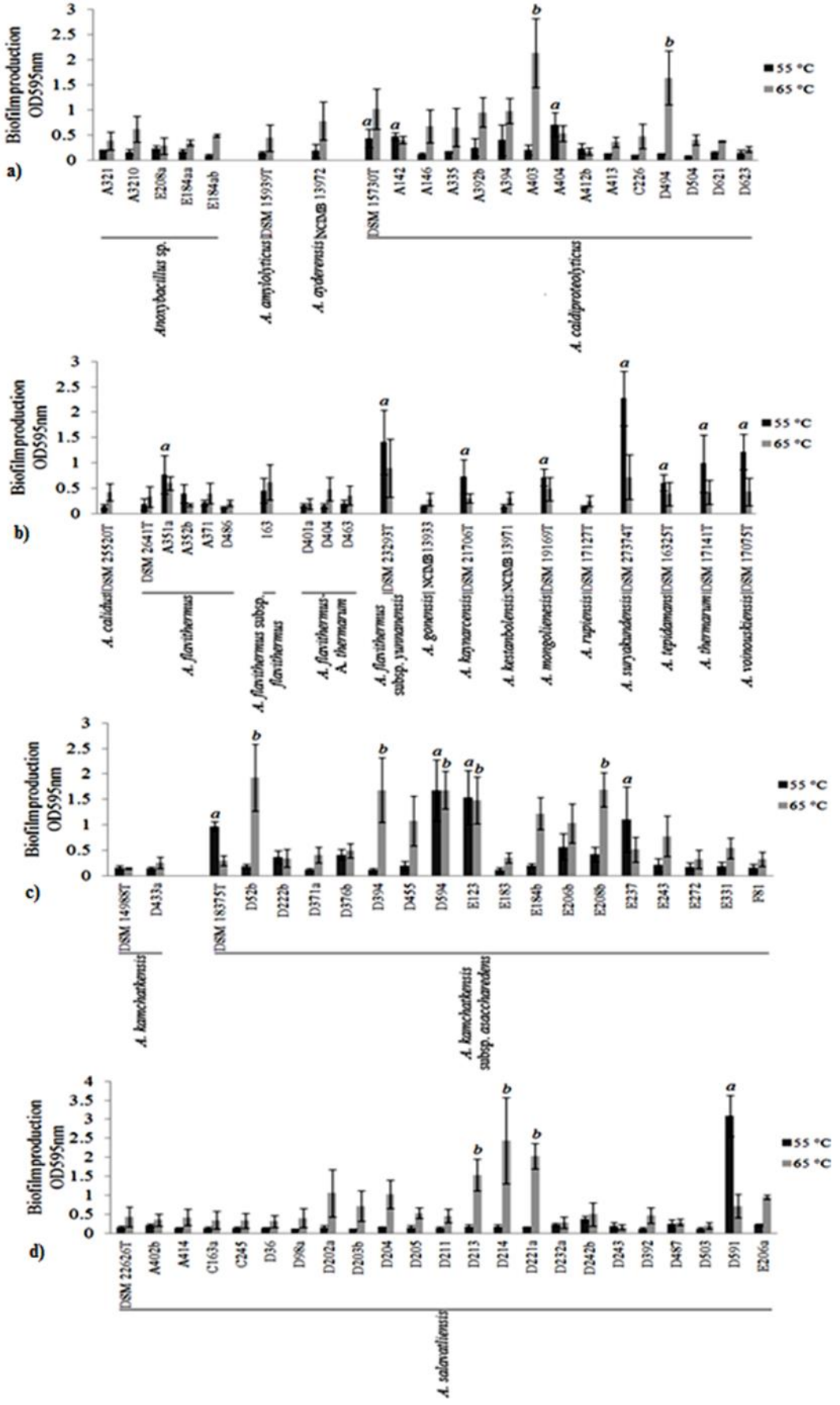

a)
Biofilm production OD595nm
55 °C
65 °C
A321
A3210
E208a
E184aa
E184ab
DSM 15730T
A142
A146
A335
A392b
A394
A403
A404
A412b
A413
C226
D494
D504
D621
D623
Anoxybacillus sp.
A. amylolyticus DSM 15939T
A. eydenensis NCIMB 13972
A. caldiproteolyticus
b)
A. calidus DSM 25520T
DSM 26411T
A351a
A352b
A371
D486
163
D401a
D404
D463
DSM 23293T
NCIMB 13933
DSM 21706T
NCIMB 13971
DSM 19169T
DSM 17127T
DSM 27374T
DSM 16325T
DSM 17141T
DSM 17075T
A. flavithermus
A. flavithermus subsp. flavithermus
A. flavithermus - A. thermarum
A. flavithermus subsp. yunnanensis
A. gonensis
A. kaynarcensis
A. kestanbolensis
A. mongoliensis
A. rupiensis
A. suryakhandensis
A. tepidamans
A. thermarum
A. voinovskiensis
c)
DSM 14988T
D433a
DSM 18375T
D52b
D222b
D371a
D376b
D394
D455
D594
E123
E183
E184b
E200b
E208b
E237
E243
E272
E331
FS1
A. kamchatkensis
A. kamchatkensis subsp. asaccharedens
d)
DSM 22626T
A402b
A414
C163a
C245
D36
D98a
D202a
D203b
D204
D205
D211
D213
D214
D221a
D232a
D242b
D243
D392
D487
D503
D591
E206a
A. salinatlantensis

Figure 4. The amounts of biofilm production among *Anoxybacillus* members in milk at 55°C, and 65°C. [a] and [b] indicates the significance in terms of biofilm formation and represents the best producers at 55°C and 65°C, respectively. a), b), c), and d) represent different *Anoxybacillus* isolates and reference strains

A. caldiprotelyticus and *A. kamchatkensis* subsp. *asaccharedens* members were generally at the forefront with strong biofilm production trends at both temperatures among genus *Anoxybacillus* (Tukey's Test; $p<0.05$). Besides, *A. caldiproteolyticus* DSM 15730^T reference strain and *A. caldiproteolyticus* A142 and A404 isolates, *A. flavithermus* subsp. *yunnanensis* DSM 23293^T, *A. kaynarcensis* DSM 21706^T, *A. mongoliensis* DSM 19169^T, *A. suryakundensis* DSM 27374^T, *A. tepidamans* DSM 16325^T, *A. thermarum* DSM 17141^T, *A. voinouskiensis* DSM 17075^T reference strains and *A. flavithermus* A351a isolate, *A. kamchatkensis* subsp. *asaccharedens* members such as D594, E123, E237 isolates and DSM 18375^T reference strain and finally *A. salavatliensis* D591 isolate were found to be the best at 55°C as shown in Figure 4 (Tukey's Test; $p<0.05$). On the other hand, when the amounts of biofilm production at 65°C were evaluated, it was observed that *A. caldiproteolyticus* A403 and D94 isolates, *A. kamchatkensis* subsp. *asaccharedens* D52b, D394, D594, E123 and E208b in addition to *A. salavatliensis* D213, D214, and D221a isolates were found to be the best producers (Tukey's Test; $p<0.05$).

The relationship found between the biofilm formation and some phenotypic traits

The relevant biochemical tests based on the utilization of lactose and casein were carried out in order to speculate about how biofilm production capacities may vary depending on the phenotypic characteristics. However, it was noteworthy that these phenotypic properties had no direct effect on the biofilm production tendencies of thermophilic bacilli (Table 2 and Table 3). The Chi-square test and *p* values were also given at the end of each analysis in Tables.

Discussion

The main focus of this study was to determine the biofilm production behaviours of thermophilic bacilli on behalf of the species level, previously isolated from different habitats, in which they were now, adapted to milk product-related environments. Increasing thermal stress is one of the most critical environmental factors that stimulate the formation of thermophilic endospore formation. Biofilm production of thermophilic bacilli is a process that begins with the attachment of both vegetative cells and spores (Flint et al., 2001a; Parkar et al., 2001). Previous studies have shown that *Geobacillus* and *Anoxybacillus* spores tend to adhere more to abiotic surfaces such as stainless steel when compared to vegetative cells. It was also understood that the sporulation process had been suppressed in *Geobacillus* and *Anoxybacillus* members at low temperatures (Flint et al., 2001a, b; Scott et al., 2007; Burgess et al., 2009; Kilic et al., 2017).

In the current study, the effect of two critical temperatures on biofilm production was investigated. While *Geobacillus* and *Anoxybacillus* members can grow well at 55°C, 65°C is a critical temperature that exposes these bacteria to thermal stress. All members of the *Geobacillus* and *Anoxybacillus* within the study displayed more intense biofilm production at 65°C due to increased thermal stress (Table 2 and Table 3). However, some strong producers could form high levels of biofilm both at two different temperatures. Considering the effect of incubation temperature on biofilm production, especially the members of *Geobacillus*, it can be concluded that the elevated incubation temperature increased endospore formation. These endospores of

thermophilic bacilli might also allow more biofilm production by triggering the attachment to the surfaces. Determination of strong biofilm production characteristics in the majority of *Anoxybacillus* members in standard whole milk also coincided with some findings in the literature. *Anoxybacilllus* members were frequently isolated from the units where dairy products are processed, especially milk powder processing units, compared to *Geobacillus* members (Ronimus et al., 2003; Yuan et al., 2012). Even among the current thermophilic bacilli species, *A. flavithermus* is one of the most frequently isolated species (Yuan et al., 2012). Based on this data, it can be concerned that *Anoxybacillus* members are more prone to biofilm production in milk-containing environments and can sustain their existence longer.

The approaches in the literature, evaluating the effects of milk components on the biofilm production behaviours of thermophilic bacilli, are mostly focused on how these components affect the bacterial attachment patterns depending on the changing surface charge. There are reports that milk components affect the attachment of microorganisms to the surfaces and biofilm production capabilities in a variety of ways. For example; *Pseudomonas* sp. that may be found in the normal flora of raw milk was found to be produced less biofilm on stainless steel surfaces by increasing the sugar and protein contents of the milk (Bernbom et al., 2009). In addition, it has been reported that the adhesion tendency of thermophilic bacilli spores and vegetative cells to the surfaces in the diluted milk was reduced (Parkar et al., 2001). The milk components in organic nature such as casein and lactose also reduced the ability of essential milk pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* to stainless steel surfaces. An additional consideration for the evaluation of this approach is to understand how the biofilm production process can be varied in the presence of lactose and casein utilizing bacteria that may be present in dairy environments.

Moreover, due to the fermentation of lactose and the hydrolysis of the casein, the released metabolites can be nutritional supplements for those bacteria which cannot hydrolyze casein or ferment lactose (Quigley et al., 2013). Therefore, we also investigated whether the lactose fermentation, the casein hydrolysis, or both had any effect on the biofilm responses of thermophilic bacilli with statistical approaches. However, it was concluded that there was no significant correlation between these phenotypic characteristics and biofilm production capacities among the tested *Geobacillus* and *Anoxybacillus* members.

Additionally, biofilm responses were determined based on the different incubation times, and consequently, the incubation period was preferred as the biofilm production reached its maximum level after 48 hours for almost all tested thermophilic bacilli. Under longer-term incubation conditions, whey proteins in standard whole milk denature and intensely accumulate on product processing surfaces at high temperatures (Flint et al., 2001a; Marchand et al., 2012). Despite the mentioned obstacle above, the biofilm production behaviours of thermophilic bacilli were evaluated at high temperatures and in whole milk according to the crystal violet binding assay, and repeatable results were obtained in the current study. This method was modified by using standard whole milk for the first time in order to quantify the amount of thermophilic biofilm production. Thereby, this approach can be preferred directly for the fast and reliable preliminary determination of the biofilm production characteristics of thermophilic bacilli in milk. The modified methodological approach proposed in this study may also be able to fill a significant gap in the literature for further studies and correspond to a principle that can consistently determine the characteristics of thermophilic biofilm production within a short period.

In conclusion, it was deduced from this study that many of the lesser-known *Geobacillus* and *Anoxybacillus* species biofilms might be as problematic as well known thermophilic bacilli biofilms in the dairy industry. In addition to some mostly isolated thermophilic bacilli species frequently originated from the dairy industry (*A. flavithermus*, *G. stearothermophilus* etc.), other thermophilic bacilli species must be taken into consideration when both assessing the risks for the dairy industry and also the new species and habitat delineation concepts altogether.

Acknowledgements

This research was supported by The Scientific and Technical Research Council of Turkey (TÜBİTAK) by the project number of 116Z422.

Compliance with ethical standards

The authors declare that they have no competing interests.

References

Bernbom, N., Ng, Y. Y., Jørgensen, R. L., Arpanaei, A., Meyer, R. L., Kingshott, P., & Gram, L. (2009). Adhesion of food-borne bacteria to stainless steel is reduced by food conditioning films. *Journal of Applied Microbiology*, 106(4), 1268-1279.

Bezuidt, O. K., Pierneef, R., Gomri, A. M., Adesioye, F., Makhalanyane, T. P., Kharroub, K., & Cowan, D. A. (2016). The *Geobacillus* pan-genome: implications for the evolution of the genus. *Frontiers in Microbiology*, 7, 723.

Burgess, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2009). The formation of spores in biofilms of *Anoxybacillus flavithermus*. *Journal of Applied Microbiology*, 107(3), 1012-1018.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215-225.

Cihan, A. C., Ozcan, B., Tekin, N., & Cokmus, C. (2011). Phylogenetic diversity of isolates belonging to genera *Geobacillus* and *Aeribacillus* isolated from different geothermal regions of Turkey. *World Journal of Microbiology and Biotechnology*, 27(11), 2683.

Claus, D., Berkeley, R. C. W., Genus *Bacillus*. 1105–1139. *Bergey's Manual of Systematic Bacteriology*, 1986, pp. 1105–1139. Williams and Williams, Baltimore. ISBN 9780683410518.

De Clerck, E., Vanhoutte, T., Hebb, T., Geerinck, J., Devos, J., & De Vos, P. (2004). Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. *Applied and Environmental Microbiology*, 70(6), 3664-3672.

Denny, C. B. (1981). Thermophilic organisms involved in food spoilage: introduction. *Journal of Food Protection*, 44(2), 144-145.

Flint, S. H., Ward, L. J., & Walker, K. M. (2001). Functional grouping of thermophilic *Bacillus* strains using amplification profiles of the 16S–23S internal spacer region. *Systematic and applied microbiology*, 24(4), 539-548.

Flint, S., Palmer, J., Bloemen, K., Brooks, J., & Crawford, R. (2001a). The growth of *Bacillus stearothermophilus* on stainless steel. *Journal of Applied Microbiology*, 90(2), 151-157.

Hayes, P. R., Quality Assurance and Production Control, 1995, In: Hayes P.R. (eds) Food Microbiology and Hygiene. Springer, Boston, MA. ISBN 978-1-4613-6574-7.

Hill, B. M., & Smythe, B. W. (2012). Endospores of thermophilic bacteria in ingredient milk powders and their significance to the manufacture of sterilized milk products: an industrial perspective. *Food Reviews International*, 28(3), 299-312.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Food protection with high temperatures, and characteristics of thermophilic microorganisms. *Modern food microbiology*, 415-441.

Kilic, T., Karaca, B., Ozel, B. P., Ozcan, B., Cokmus, C., & Coleri Cihan, A. (2017). Biofilm characteristics and evaluation of the sanitation procedures of thermophilic *Aeribacillus pallidus* E334 biofilms. *Biofouling*, 33(4), 352-367.

Koc, M., Cokmus, C., & Cihan, A. C. (2015). The genotypic diversity and lipase production of some thermophilic bacilli from different genera. *Brazilian Journal of Microbiology*, 46(4), 1065-1076.

Konstantinidis, K. T., Ramette, A., & Tiedje, J. M. (2006). The bacterial species definition in the genomic era. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1475), 1929-1940.

Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L. (2012). Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 133-147.

Parkar, S. G., Flint, S. H., Palmer, J. S., & Brooks, J. D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology*, 90(6), 901-908.

Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., & Cotter, P. D. (2013). The complex microbiota of raw milk. *FEMS Microbiology Reviews*, 37(5), 664-698.

Ronimus, R. S., Parker, L. E., Turner, N., Poudel, S., Rückert, A., & Morgan, H. W. (2003). A RAPD-based comparison of thermophilic bacilli from milk powders. *International Journal of Food Microbiology*, 85(1-2), 45-61.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, 60(2), 109-117.

Stepanović, S., Vuković, D., Dakić, I., Savić, B., & Švabić-Vlahović, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40(2), 175-179.

Suzuki, Y., Kishigami, T., Abe, S. (1976). Production of extracellular alpha-glucosidase by a thermophilic *Bacillus* species, *Applied and Environmental Microbiology*, 31(6), 807-812.

Vert, M., Doi, Y., Hellwich, K. H., Hess, M., Hodge, P., Kubisa, P, & Schué, F. (2012). Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). *Pure and Applied Chemistry*, 84(2), 377-410.

Vestby, L. K., Møretrø, T., Langsrud, S., Heir, E., & Nesse, L. L. (2009). Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. *BMC veterinary research*, 5(1), 20.

Yuan, D. D., Liu, G. C., Ren, D. Y., Zhang, D., Zhao, L., Kan, C. P., ... & Zhang, L. B. (2012). A survey on occurrence of thermophilic bacilli in commercial milk powders in China. *Food Control*, 25(2), 752-757.