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Research Note

Quantities of Adenylate Homologues (ATP+ADP+AMP) Change over Time in Prokaryotic and Eukaryotic Cells

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ABSTRACT

Rapid assays for the assessment of the hygienic state of surfaces in food and medical industries include the use of technologies designed to detect the presence of the metabolite ATP. ATP is a critical metabolite and energy source for most living organisms; therefore, the presence of ATP can be an indicator of surface hygiene based on the presence of soil or food residues associated with inadequate cleaning. The concentrations of ATP vary based on an organism's metabolic state, thus potentially influencing the sensitivity of ATP-based assays. However, little has been published detailing the quantitative changes of ATP to the adenylate homologues ADP and AMP nor the quantitative and cumulative fate of these homologues over time as the metabolic state remains in flux. The objective of this study was to quantify the individual and cumulative (AXP) concentrations of these three adenylate homologues over defined time periods in selected eukaryotic tissue and prokaryotic cell cultures of significance to hygiene. ATP concentrations differed substantially across these selected variables of time and source. The 1- to 3-log reductions in ATP concentrations over time were highly affected by organism type. In general, ADP became the predominate adenylate in eukaryotic tissue, and AMP was the predominate adenylate in the prokaryotic cells at later time points in each study. Total AXP concentrations dropped in general, reflective primarily of the loss of ATP. The results of ATP-based techniques for hygiene surveillance will vary as a function of the amount of cellular material present and the metabolic state of such material.

HIGHLIGHTS

- ATP concentrations varied as a function of time and source in eukaryotic and prokaryotic samples.
- ADP became the predominant homologue over time in eukaryotic samples tested.
- AMP became the predominant homologue over time in prokaryotic samples tested.
- Hygiene assessment methods based on ATP alone may not account for changes in adenylate homologues.

Key words: ATP; Hygiene; Rapid assay; Sanitation

The safe and legal manufacture of food in the United States and many other countries is being increasingly scrutinized to control and verify critical processes known to influence contamination *(8)*. The ability to rapidly collect objective data regarding the hygienic state of direct food contact surfaces is especially critical *(5, 14)*. For this purpose, food processing companies often employ ATP- or bioluminescence-based technologies to swab for and assess the hygiene of manufacturing plant surfaces *(11–13, 17)*. Such technologies provide convenient and rapid assessments of contamination on food contact surfaces and in other areas affecting overall plant hygiene *(6, 21)*. One concern regarding an ATP-based assay is that the concentration of ATP in cellular material changes based on the metabolic state of the cell, which can be affected under conditions of nutrient depletion or other environmental stressors *(20)*. These conditions can occur in food processing plants and can result in changes to cellular metabolism wherein the native pool of ATP is dephosphorylated into ADP or AMP *(1–3, 7)*. Because such metabolically based phenomena can result in significant changes in ATP concentrations, the sensitivity of ATP-based hygiene assessment technologies may be reduced *(4),* thus increasing the risk or rate of false-negative results.

Prokaryotic cellular material and eukaryotic animal tissue are two important sources of ATP contamination generally for food processing and specifically for meat processing facilities; however, few applied or basic studies have been conducted to quantify the fundamental changes in ATP concentrations as a function of time or metabolic state *(9)*. In the present study, ATP, ADP, and AMP (cumulatively referred to as AXP) were quantified under defined conditions of holding time and temperature for bovine, porcine, and poultry tissues and four bacterial cell cultures of significance to food hygiene: *Escherichia coli, Crono-*

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bacter sakazakii, Lactobacillus casei, and *Bacillus subtilis*. The objective of this work was to quantify changes in ATP concentrations under the defined conditions and times reasonably encountered in a food processing environment.

MATERIALS AND METHODS

AXP quantification. All samples were analyzed using a bioluminescent luciferin-luciferase assay. Measurements of ADP and AMP were carried out using pyruvate kinase and pyruvate phosphate dikinase, respectively. Concentrations of adenylate homologues were calculated against curves of authentic adenylate standards *(4, 15)*. Consumable chemicals were luciferase reagents and adenosine phosphate deaminase (Kikkoman Biochemifa, Tokyo, Japan); reagent grade ATP, ADP, and AMP (Oriental Yeast, Tokyo, Japan); and trichloroacetic acid, EDTA, and reagent grade water (Fisher Scientific, Hampton, NH). Bioluminescent measurements were performed with a luminometer (Lumitester C-110, Kikkoman Biochemifa). Readings are reported as an average of 10 individual 1-s measurements, each of which has a luminescence signal cumulated over each 1-s interval. The device has a relative light unit (RLU) range of 0 to 999,999.

Eukaryotic sample preparation. Muscle tissue samples from animal carcasses were obtained from the University of Wisconsin–Madison's Meat Science and Muscle Biology Laboratory. No animals were slaughtered or otherwise used specifically for the purpose of this research beyond the normal activity of the Laboratory. Bovine and porcine samples were aseptically collected from the hindquarters of the respective carcasses. Bovine samples were collected from the biceps femoris muscle, and porcine samples were collected from the semimembranosus muscle. Poultry samples were collected from the pectoral muscle. Before sampling at each time point, the exposed surface area of the muscle was aseptically removed, and the sample was collected from a previously unexposed portion of each carcass. Preliminary work was conducted to confirm that samples collected in this way did not contain microbial contaminants (data not shown).

AXP analysis on eukaryotic samples. Two grams of muscle sample was added to 8 mL of trichloroacetic acid (TCA) extraction solution (10% TCA plus 4 mM EDTA·disodium salt; Fisher Scientific, Hampton, NH). Duplicate 2-g portions were analyzed from each meat sample at each time point. These subsamples were extracted in the TCA solution for 30 min and then diluted with 25 mM tricine-NaOH buffer (pH 7.75; Fisher Scientific) treated with adenosine phosphate deaminase. Aliquots (0.1 mL) of the final dilution were added to 0.1 mL of each luciferin-luciferase reagent (Kikkoman Biochemifa) in an adenylate-free plastic tube (Lumitube, Kikkoman Biochemifa), vortexed briefly, and analyzed for luminescence with a luminometer (Lumitester C-110). Two RLU measurements were made for each sample replicate and averaged for analysis. Sample duplicates were averaged and calculated for adenylate concentrations using standard curves of authentic standards of ATP, ADP, and AMP from stock solutions prepared with a range of 1×10^{-11} to 1×10^{-7} mol/mL.

Microorganisms. *C. sakazakii* ATCC 29544, *E. coli* ATCC 25922*, L. casei* ATCC 393, and *B. subtilis* ATCC 6051 (Microbiologics, St. Cloud, MN) were selected for study based on their presence as contaminants in food processing venues. Phosphate buffer solution (PBS) and microbial media were obtained from a commercial source (Fisher Scientific).

E. coli was inoculated with a microbial swab (KWIK-STIK, Microbiologics) into 10 mL of tryptic soy broth (TSB; Fisher Scientific) 22 to 24 h before the AXP analysis. At the beginning of the analysis, stock cultures were diluted to an optical density at 600 nm (OD_{600}) of approximately 0.001 with TSB. Tryptic soy agar plates were used for enumeration. Plates and cultures were incubated at 37°C. Cells were harvested from the culture at 12,000 $\times g$ for 1 min and washed with PBS. Cells were washed twice with PBS and then used for TCA extraction and enumeration.

C. sakazakii was inoculated into 10 mL of sterile nutrient broth (BD nutrient broth, Fisher Scientific) 22 to 24 h before AXP analysis. At the beginning of the analysis, stock cultures were diluted to an OD_{600} of approximately 0.01 with nutrient broth. Nutrient agar plates were used for enumeration. Cultures were incubated at 30°C. Cells were harvested by centrifugation at 5,000 $\times g$ for 5 min and resuspended with PBS. Cells were washed twice with PBS and then used for TCA extraction and enumeration.

L. casei was inoculated into 10 mL of de Man Rogosa Sharpe (MRS) broth (Fisher Scientific). After approximately 48 h of incubation at 37°C, stock cultures were diluted to an OD_{600} of approximately 0.001 and allowed to incubate overnight at 37°C. AXP analysis began the following morning. MRS agar plates were used for enumeration and incubated at 37°C. The atmosphere was not modified during this experiment. Cells were harvested at 12,000 $\times$ g for 1 min and resuspended with PBS. Cells were washed twice with PBS and then used for TCA extraction and enumeration.

B. subtilis was inoculated into 4 mL of nutrient broth. Cultures were shaken at 190 rpm at 37°C. After 18 h, stock cultures were adjusted with nutrient broth to an OD_{600} of approximately 0.1. Cultures were grown in 250-mL glass Erlenmeyer flasks that were shaken at 190 rpm at 37°C. Nutrient agar plates were used for enumeration and incubated at 37°C. Cells were harvested at 12,000 $\times g$ for 1 min and resuspended with PBS. Cells were washed twice with PBS and then used for TCA extraction and enumeration.

Microbial AXP measurements. After the cells were washed twice with PBS and a subsample was removed for enumeration, the remaining suspension was centrifuged and the pellet was resuspended with TCA solution for 30 min and then diluted 100- to 1,000-fold with dilution buffer. A 0.1-mL aliquot of the diluted sample was mixed with 0.1 mL of the appropriate luciferase reagent for analysis.

Design of experiment and statistical analysis. Each of the main adenylate sources (eukaryotic animal tissue or prokaryotic microbial cells) was collected minimally in triplicate. Samples were analyzed at a minimum of five time points for each bovine, porcine, and poultry sample, and three carcasses of each animal species were assessed. The date of AXP concentration analysis was included as a block in the initial statistical analysis with time as the main treatment variable, but date of analysis was removed from the final ANOVA because it did not have a significant effect. The final statistical design and data analysis were conducted using statistical software (JMP v. 14, SAS Institute, Cary, NC), and ANOVA outputs are reported in Tables 1 through 3.

RESULTS AND DISCUSSION

For both bovine and porcine (Fig. 1) samples, an approximately 3-log decrease in ATP concentrations was found within 48 h of slaughter. ADP concentrations remained relatively constant over the time of analysis,

TABLE 1. *Summary of ANOVA results of AXP concentrations for selected eukaryotic organisms*

Organism	Source	Degrees of freedom	Sum of squares	*F* ratio	*P* value
Porcine	ATP	4	4.93×10^{-13}	29.98	<0.0001
	ADP	4	1.33×10^{-15}	1.54	0.2227
	AMP	4	2.19×10^{-16}	0.6	0.6677
	AXP	4	5.49×10^{-13}	30.57	<0.0001
Poultry	ATP	4	5.30×10^{-13}	33.55	<0.0001
	ADP	4	4.70×10^{-15}	3.78	0.0167
	AMP	4	8.13×10^{-16}	1.22	0.3298
	AXP	4	6.13×10^{-13}	27.92	<0.0001
Bovine	ATP	12	5.90×10^{-13}	30.61	<0.0001
	ADP	12	1.85×10^{-15}	1.45	0.2073
	AMP	12	1.37×10^{-15}	2.13	0.0515
	AXP	12	6.40×10^{-13}	24.72	<0.0001

but a 1-log reduction in AMP concentrations was noted. Total AXP concentrations in bovine tissue fell from an average of 3.0×10^{-7} mol/g at slaughter to 3.8×10^{-8} mol/g after 48 h. Porcine samples displayed similar trends over the first 24 h postslaughter, with a decline in total average AXP from 4.2×10^{-7} to 4.1×10^{-8} mol/g. ADP was the primary adenylate compound present after 48 h in both porcine and bovine carcasses. A 2-log reduction in ATP was measured for poultry (Fig. 1) within 26 h of slaughter. ADP concentrations were constant over the time of analysis, but a 1-log reduction in AMP concentrations was noted. Overall AXP concentrations in poultry fell from an average of 3.5×10^{-7} mol/g at slaughter to 3.8×10^{-8} mol/g after 26 h. ADP was the primary adenylate compound present after 6 h in poultry carcasses. For all eukaryotic species, changes in AXP and ATP concentrations were significant over the time of analysis (Table 1). Declines in ATP concentrations during rigor mortis and aging likely explain the change in total AXP concentrations. Changes in ADP and AMP concentrations over the time of analysis were less significant.

E. coli and *C. sakazakii* (Fig. 2) had similar growth and AXP profiles. Peak levels (CFU per milliliter) were reached by 24 h following inoculation into fresh media, where the initial OD_{600} for *E. coli* was 0.001 and the initial OD_{600} for *C. sakazakii* was 0.01 (Fig. 3). Viable cell counts remained near peak values for 7 to 14 days before declining. Total AXP concentrations reached a maximum of 3.4×10^{-9} mol/mL at 84 h for *C. sakazakii* and 7.7×10^{-9} mol/mL at 48 h for *E. coli*. AMP was the predominate adenylate compound present at the majority of the time points analyzed.

L. casei peak levels (CFU per milliliter) were reached by 48 h following inoculation into fresh media, where the initial OD_{600} was 0.1 (Fig. 3). Viable cell counts remained near peak values through 48 h before declining. Total AXP concentrations reached a maximum of 1.8×10^{-8} mol/mL at 48 h (Fig. 2). ATP initially was the predominate adenylate following the initial OD adjustment and then declined through day 6 of evaluation. AMP was the predominate adenylate compound at the majority of the time points analyzed.

B. subtilis peak levels (CFU per milliliter) were reached by 8 h following inoculation into fresh media, where the initial OD_{600} was 0.1 (Fig. 3). Viable cell count remained near peak values through 6 h before declining. Total AXP concentrations reached a maximum of 8.5×10^{-10} mol/mL at 24 h (Fig. 2). ATP initially was the predominate adenylate following the initial OD adjustment. After 1 h of growth, AMP became the predominate adenylate and remained so throughout the remainder of the study.

As expected, prokaryotic organisms had different growth curves, with *E. coli* and *C. sakazakii* maintaining viable cell counts for 2 or more weeks before levels (CFU

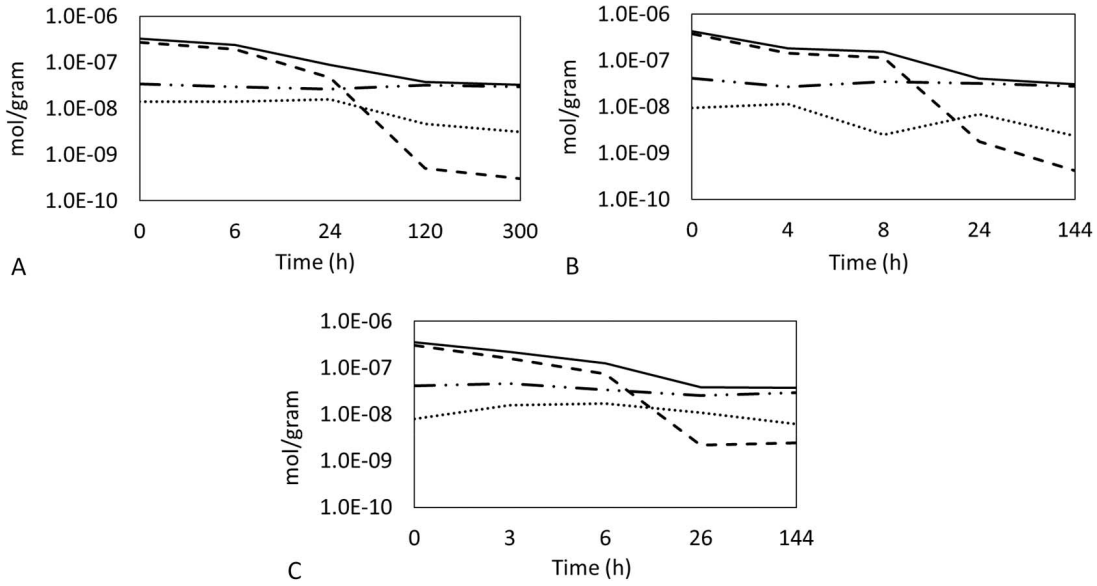

FIGURE 1. *Average concentrations of total adenylate (AXP, solid line) and adenylate homologues (ATP, dashed line; ADP, dashed-dotted line; AMP, dotted line) postslaughter as a function of time. Sampling began at time of slaughter and continued until day of fabrication. (A) Bovine samples; (B) porcine samples; (C) poultry samples.*

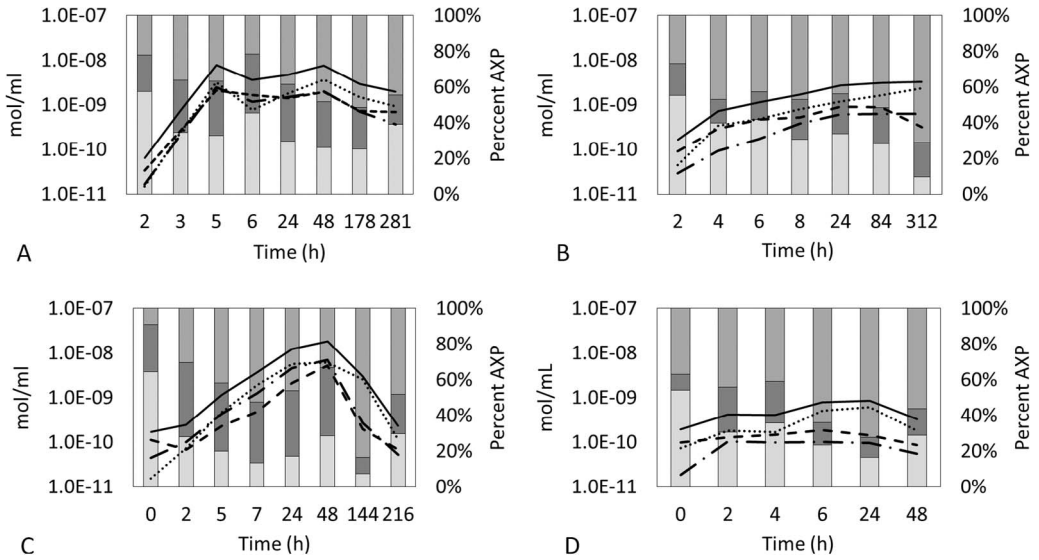

FIGURE 2. *Average concentrations of total adenylate (AXP, solid line) and adenylate homologues (ATP, dashed line; ADP, dashed-dotted line; AMP, dotted line) postslaughter as a function of time. Following overnight incubation, initial cell levels were adjusted using* OD_{600} *values. Measurements for AXP concentrations started after cell level adjustments and continued until a minimum 1-log reduction in the average level of viable organisms was obtained. %ATP, light shading; %ADP, dark shading; %AMP, medium shading. (A)* E. coli*; (B)* C. sakazakii*; (C)* L. casei*; (D)* B. subtilis.

per milliliter) began to decline. *L. casei* reached the death phase approximately 1 week after the initial OD adjustment. *L. casei* also had the highest total AXP concentration per milliliter compared with the other three organisms (*E. coli, C. sakazakii, L. casei,* and *B. subtilis*: 7.7×10^{-9}, 3.4×10^{-9}, 1.8×10^{-8}, and 8.9×10^{-10} mol/mL, respectively). *B. subtilis* had the lowest overall AXP concentration but also had the shortest growth cycle and generally the lowest counts (CFU per milliliter). Some of these differences are due to each microorganism's unique metabolic pathways and cellular growth properties under the conditions present in this study. *L. casei* had a much greater cell mass than did the other bacteria in this study, possibly because of its ability to ferment carbon substrate more effectively than respiring organisms. This ability could also lead to an earlier transition to the death phase as carbon substrates are depleted and waste products accumulate in the culture. *B. subtilis* has defense mechanisms, such as sporulation and biofilm formation, that can be used during stress. Cannibalistic activity precedes sporulation and biofilm formation and is used to delay the onset of either response. These mechanisms may further explain the declining OD_{600} values, lower peak cell levels, and lower adenylate production compared with the other three microbial species

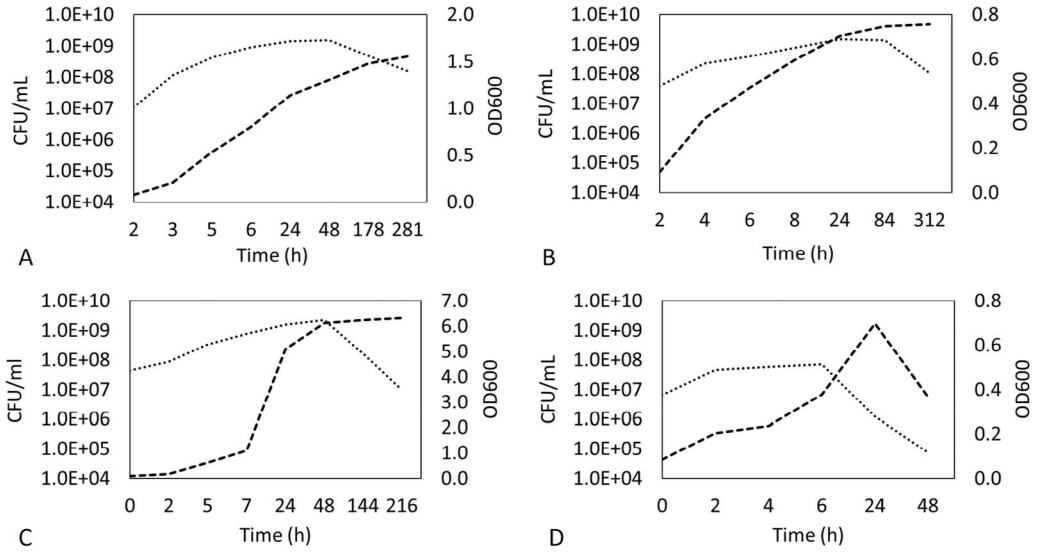

FIGURE 3. *Average level of viable organisms (CFU per milliliter, dotted line) and optical density (*OD_{600}*, dashed line) of inoculum as a function of time. Samples were collected at time of AXP measurement and enumerated by plating. (A)* E. coli*; (B)* C. sakazakii*; (C)* L. casei*; (D)* B. subtilis.

TABLE 2. *Summary of ANOVA results of AXP concentrations for selected prokaryotic microorganisms*

Microorganism	Source	Degrees of freedom	Sum of squares	*F* ratio	*P* value
E. coli	ATP	14	3.77×10^{-17}	7.84	<0.0001
	ADP	14	4.12×10^{-17}	3.6	0.0009
	AMP	14	1.03×10^{-16}	4.51	0.0001
	AXP	14	4.85×10^{-17}	4.79	<0.0001
B. subtilis	ATP	5	1.94×10^{-20}	4.07	0.019
	ADP	5	2.69×10^{-20}	5.82	0.0049
	AMP	5	9.54×10^{-23}	23.48	<0.0001
	AXP	5	1.40×10^{-18}	19.38	<0.0001
C. sakazakii	ATP	11	3.44×10^{-18}	34.96	<0.0001
	ADP	11	4.31×10^{-18}	27.39	<0.0001
	AMP	11	2.24×10^{-17}	85.73	<0.0001
	AXP	11	6.06×10^{-17}	175.95	<0.0001
L. casei	ATP	8	8.67×10^{-17}	35.97	<0.0001
	ADP	8	1.57×10^{-16}	39.42	<0.0001
	AMP	8	1.96×10^{-16}	50.82	<0.0001
	AXP	8	1.13×10^{-15}	604.34	<0.0001

(19). Adenylate homologue and total AXP concentrations differed significantly by time and growth stage of each microbial species (Table 2). Changes in AXP concentrations were likely influenced by the number of live cells in the culture (Table 3).

ATP-based hygiene assessments rely on the principle that surface contaminants contain ATP in concentrations sufficient for detection and that changes in ATP, and thus assessment values, are reflective of the degree of residual contamination or the hygienic state, e.g., following a cleaning protocol *(16)*. Concentrations of ATP (and thus hygienic assessment values) ideally would remain consistent over time as long as the ATP-containing soils remain in place *(18)*. However, results of the present study have revealed that ATP concentrations can vary by several orders of magnitude either the eukaryotic tissue or prokaryotic contaminants continue to utilize ATP. This work provides quantitative data that under the specific conditions described in these studies the profiles of these adenylate homologues shift from ATP, and ADP and AMP become the predominant adenylate species *(10)*. This work also indicates that the cumulative pool of adenylate (AXP) is also in flux and correspondingly susceptible to depletion over time. However, because AXP concentration is a function of the concentrations of all three homologues, it remains present in concentrations that are significantly higher than those of ATP alone. The final predominant adenylate compound was different in the eukaryotic and prokaryotic cells. AMP was the predominate adenylate in the prokaryotic cells, whereas ADP was predominate adenylate in the animal cells. When considering the results of ATP-based hygiene assessments, researchers should consider that variables such as time, cellular source, and species may alter the quantity of ATP available for such assays, thus resulting in a corresponding change in the assay limit of detection.

TABLE 3. *Summary of ANOVA results for population measures of selected prokaryotic microorganisms*

Microorganism	Measure	Degrees of freedom	Sum of squares	*F* ratio	*P* value
E. coli	CFU/mL	14	1.82×10^{19}	18.34	<0.0001
	OD_{600}	14	17.27	85.01	<0.0001
C. sakazakii	CFU/mL	11	1.33×10^{19}	48.79	<0.0001
	OD_{600}	11	3.75	1,284.38	<0.0001
L. casei	CFU/mL	8	1.70×10^{19}	53.99	<0.0001
	OD_{600}	8	232.334	907.19	<0.0001
B. subtilis	CFU/mL	5	1.81×10^{16}	34.96	<0.0001
	OD_{600}	5	0.895	106.75	<0.0001

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