Supplementary Information

Rapid Detection and Strain-Level Identification of Milk-borne Bacteria Using a Polymer-Based Chemical Tongue

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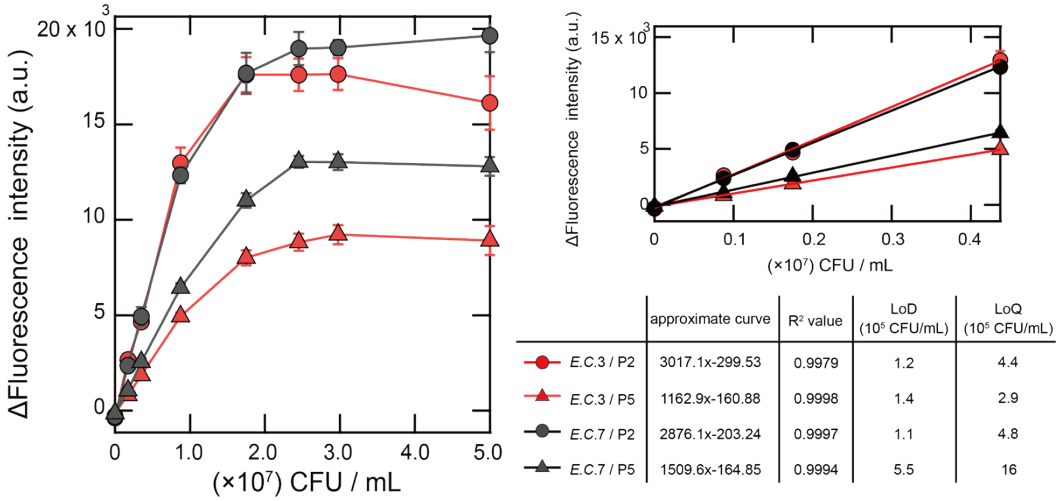
Synthesis

Dansylated-polymers (Dnc-polymers) were prepared according to slightly modified literature procedures.[1] **P1**: A solution of Dnc-Cl (8.4 mg, 31.3 μmol) in THF (1.6 mL) was quickly added to a stirred solution of PLL_{10} (50.0 mg, 208.3 μmol for amino groups) and triethylamine (145.2 μL, 1042 μmol) in methanol (6.3 mL) at room temperature. **P2**: A solution of Dnc-Cl (25.1 mg, 93.0 μmol) in THF (4.7 mL) was quickly added to a stirred solution of PLL_{55} (150.0 mg, 620.3 μmol for amino groups) and triethylamine (432.3 μL, 3102 μmol) in methanol (18.8 mL) at room temperature. **P3**: A solution of Dnc-Cl (8.4 mg, 31.0 μmol) in THF (1.6 mL) was quickly added to a stirred solution of PLL_{258} (50.0 mg, 206.7 μmol for amino groups) and triethylamine (144.1 μL, 1034 μmol) in methanol (6.3 mL) at room temperature. **P4**: A solution of Dnc-Cl (8.6 mg, 31.7 μmol) in THF (1.6 mL) was quickly added to a stirred solution of PEG_{114}-*b*-PLL_{52} (70.0 mg, 211.6 μmol for amino groups) and triethylamine (147.5 μL, 1058 μmol) in methanol (8.8 mL) at room temperature. **P5**: A solution of Dnc-Cl (3.4 mg, 12.6 μmol) in THF (0.6 mL) was quickly added to a stirred solution of PAMAM dendrimer (20.0 mg, 90.1 μmol for amino groups) and triethylamine (62.8 μL, 450 μmol) in methanol (5.0 mL) at room temperature. The reaction mixtures were stirred for 24 hours at room temperature and then dialyzed against: 1) 10% methanol (2 h), 2) deionized water (2 h), 3) 1 mM aqueous HCl (overnight), 4) 1 mM aqueous HCl (2 h), and 5) deionized water (2 h). The final solutions were lyophilized to obtain **P1**–**P5** as the chloride salt. **P2–P4** were obtained in high isolated yields (typically >90% by mass recovery), whereas **P1** and **P5** were obtained in moderate yield (approximately 60% and 40%, respectively). The number of Dnc moieties conjugated to each polymer was determined from the absorbance at 330 nm (Abs_{330}) of the obtained polymers in deionized water, using the equation [Dnc] = (Abs_{330}/4570); 1.2 (**P1**), 6.6 (**P2**) 28.5 (**P3**), 6.2 (**P4**), and 7.6 (**P5**) Dnc moieties were conjugated to each polymer.

Figures and Tables



	approximate curve	R^2 value	LoD (10^5 CFU/mL)	LoQ (10^5 CFU/mL)
*E.C.*3 / P2	3017.1x-299.53	0.9979	1.2	4.4
*E.C.*3 / P5	1162.9x-160.88	0.9998	1.4	2.9
*E.C.*7 / P2	2876.1x-203.24	0.9997	1.1	4.8
*E.C.*7 / P5	1509.6x-164.85	0.9994	5.5	16

Fig. S1. Fluorescence response curves (ΔFluorescence intensity vs CFU/mL) for **P2** and **P5** (20 μg mL^{-1}) upon addition of *E.C.*3 and *E.C.*7 in 20 mM MOPS buffer (pH = 7.0); $\lambda_{ex}/\lambda_{em}$ = 330 nm/480 nm. The linear dynamic range (shown in the inset) was determined from the linear portion of the response curve. The limit of detection (LOD) and limit of quantification (LoQ) were calculated as the concentration corresponding to the mean blank signal plus 3.3 times and 10 times its standard deviation, respectively; values shown represent mean values ± 1 SE from three independent experiments.

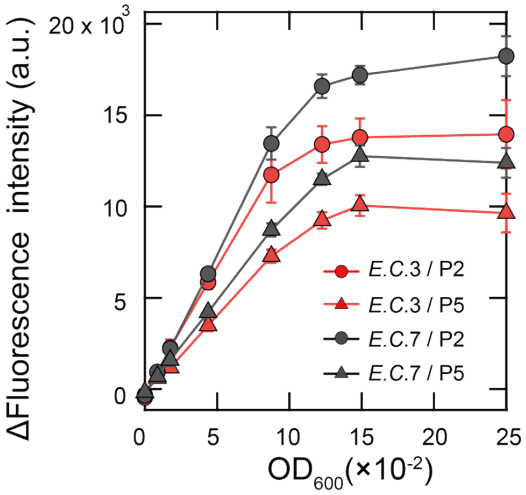


Fig. S2. Characterization of representative Dnc-polymers under mildly acidic conditions. Binding isotherms for **P2** and **P5** (20 μg mL^{-1}) upon addition of *E.C.*3 and *E.C.*7 in 20 mM acetate buffer (pH = 5.0); $\lambda_{ex}/\lambda_{em}$ = 330 nm/480 nm; values shown represent mean values ± 1 SE from three independent experiments.

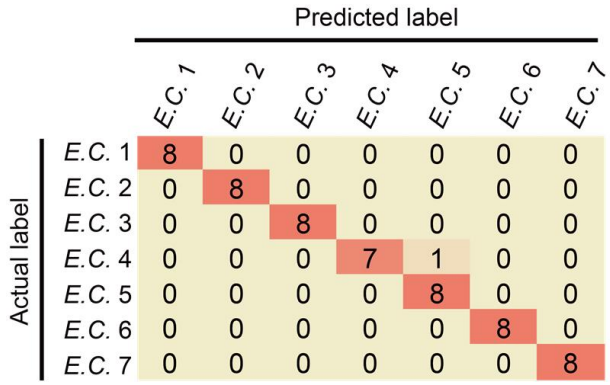


Fig. S3. Confusion matrix for the LOO cross-validation corresponding to Fig. 3. The overall classification accuracy was 98% (55/56), with a 95% confidence interval of 90.6–99.7%, calculated as a Wilson binomial proportion interval.

Table S1. Standardized coefficients for LD1 and LD2 obtained from LDA corresponding to Fig. 3. Coefficients are standardized by within-class variance. Features with relatively large absolute coefficients (highlighted in red) indicate probe–condition combinations that contribute more strongly to discrimination along each discriminant axis.

	pH = 7.0										pH = 5.0									
	P1		**P2**		**P3**		**P4**		**P5**		**P1**		**P2**		**P3**		**P4**		**P5**	
	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2	Ch1	Ch2
LD1 coefficient	1.24	-2.06	0.42	-0.23	**4.04**	-3.67	0.43	0.27	0.70	-0.32	3.70	-3.98	**4.17**	**-4.29**	0.16	-0.13	-2.78	3.02	**-4.09**	**4.70**
LD2 coefficient	1.27	-1.09	-1.63	1.49	-2.07	2.09	**-3.26**	**2.59**	**2.93**	**-2.94**	-1.86	1.96	**2.63**	-2.28	1.70	-1.62	1.20	-1.43	-0.33	0.72

Screening of pretreatment conditions

To establish a simple yet effective pretreatment to remove colloidal milk components from samples contaminated with *E. coli*, we examined two parameters, i.e., pH adjustment and NaCl supplementation (Table S2). Under the control conditions (No. 1), milk was simply diluted with distilled water and subjected to four cycles of centrifugation and pellet resuspension; this produced a large casein-micelle pellet (Fig. S4A). Using this result as a benchmark, we observed the extent of pellet formation when the distilled water was replaced with buffers of different pH (50 mM MES (pH = 5.7) or 50 mM MOPS (pH = 7.0)) with/without the addition of 200 mM NaCl.

When buffer alone was added (samples No. 3 (MES) and No. 5 (MOPS)), the pellet volume decreased markedly compared to that of sample No. 1, with MES giving the more pronounced reduction (Fig. S4A). Comparatively, the addition of 200 mM NaCl (No. 2) reduced the pellet size even further. The reduction in size when NaCl addition was combined with buffers appeared similar by eye (No. 4 and No. 6). Since the pellet appearance alone did not enable the unequivocal choice of optimum conditions, we next evaluated the increase in the fluorescence of Dnc-polymer **P5** after pretreating milk (with and without *E. coli* strain *E.C.*3) under each pretreatment condition (Fig. S4B). For milk without *E. coli*, the fluorescence increase, which reflects interactions between the residual milk components and **P5**, generally correlated with the pellet size (No. 1 ≈ No. 5 > No. 3 ≈ No. 6 > No. 2 > No. 4). This trend suggested that condition No. 4 removed milk-derived components most effectively. Indeed, for *E.C.*3-contaminated milk, the fluorescence difference between contaminated and uncontaminated samples was greatest under conditions No. 4, indicating that these conditions best extract bacterial information. Accordingly, we adopted conditions No. 4 for all subsequent analyses of *E. coli* strain detection in milk samples.

Table S2. Combination of pretreatment buffer and addition of salt solution for removal of milk-derived components.

No.	200 mM NaCl	50 mM MES (pH = 5.7)	50 mM MOPS (pH = 7.0)
1	—	—	—
2	✓	—	—
3	—	✓	—
4	✓	✓	—
5	—	—	✓
6	✓	—	✓

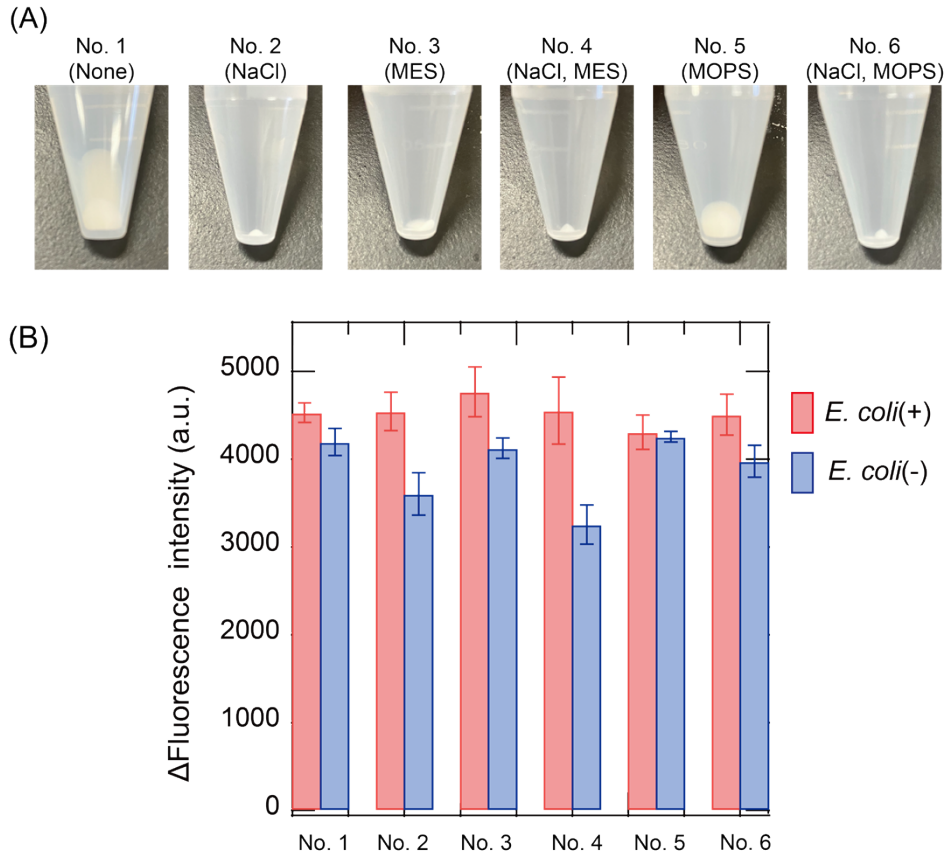


Fig. S4. Optimization of pretreatment conditions for the removal of milk-derived components. (A) Photographs of pellets obtained after centrifugation under the six conditions listed in Table S2. (B) Fluorescence changes of **P5** (20 μg mL^{-1}) in 20 mM MOPS (pH = 7.0) for milk samples pretreated under each set of conditions, measured in the absence (blue) or presence (red) of *E. coli* strain *E.C.*3 (OD_{600} = 0.1); $\lambda_{ex}/\lambda_{em}$ = 330 nm/480 nm; values shown represent mean values ±1 SE from three independent experiments.

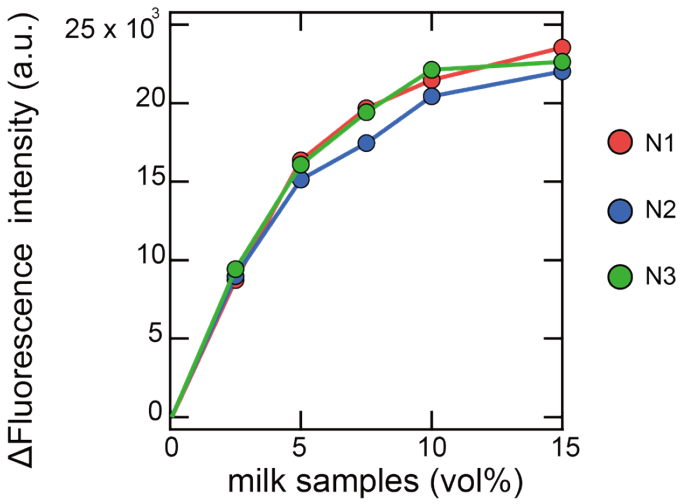


Fig. S5. Reproducibility of the pretreatment protocol. Three independently prepared milk samples (No. 4) were mixed with 20 μg mL^{-1} **P5** in 20 mM MOPS (pH = 7.0). Fluorescence intensity ($\lambda_{ex}/\lambda_{em}$ = 330 nm/480 nm) is plotted as a function of the volume fraction of the pretreated sample.

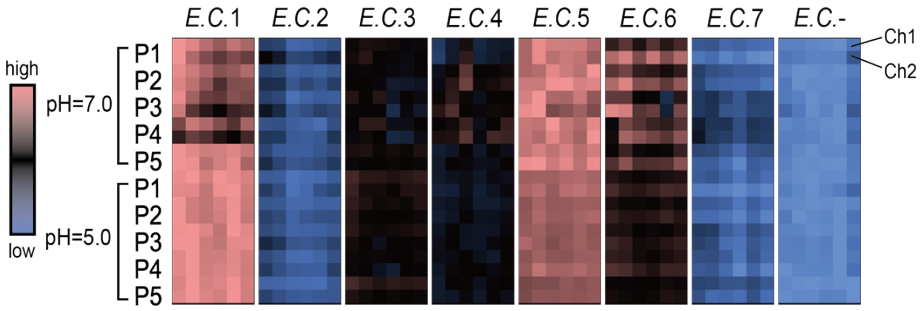


Fig. S6. Heatmap of the fluorescence -response patterns of milk samples spiked with the seven different *E. coli* strains (OD_{600} = 0.1). Four independent experimental values are shown for each analyte; each replicate is the mean of two measurements. Unspiked milk (no *E. coli*) is shown as "*E.C.*-".

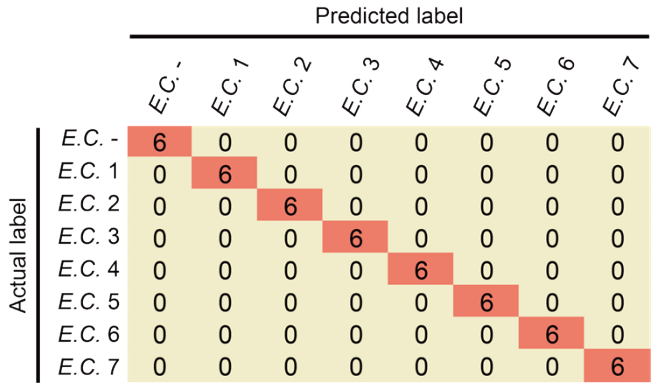


Fig. S7. Confusion matrix for the LOO cross-validation corresponding to Fig. 4D. The overall classification accuracy was 100% (48/48), with a 95% confidence interval of 92.6–100%, calculated as a Wilson binomial proportion interval

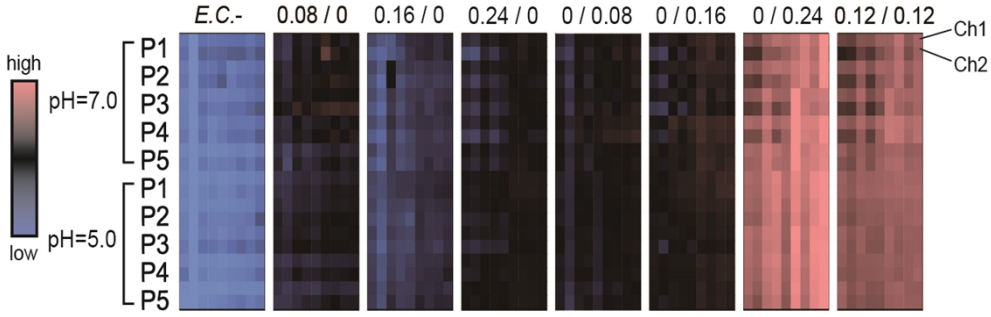


Fig. S8. Heatmap of the fluorescence-response patterns of milk samples spiked with mixtures of *E.C.*1 and/or *E.C.*5 (total OD_{600} = 0.08, 0.16, 0.24). Labels above each column indicate the OD_{600} values of *E.C.*1 and *E.C.*5, respectively (*E.C.*1 / *E.C.*5). Unspiked milk (no *E. coli*) is shown as "*E.C.*-".

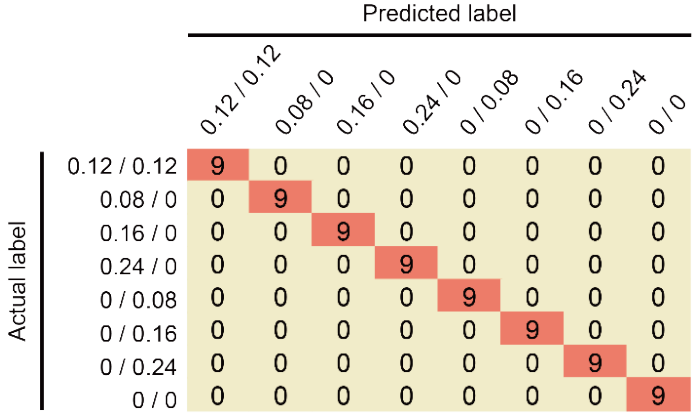


Fig. S9. Confusion matrix for the LOO cross-validation corresponding to Fig. 4E. The overall classification accuracy was 100% (72/72), with a 95% confidence interval of 94.9-100%, calculated as a Wilson binomial proportion interval.

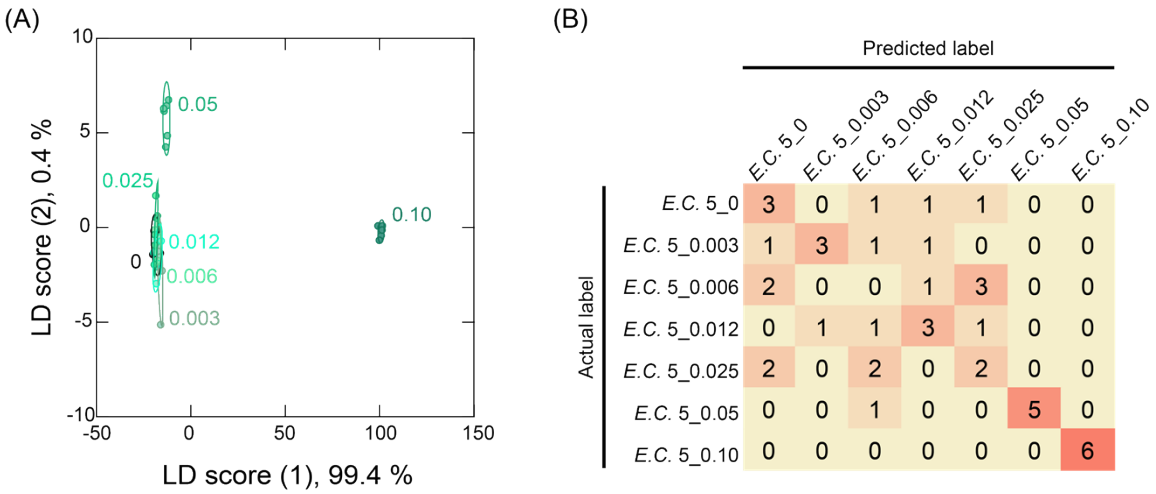


Fig. S10. (A) LDA score plots for milk samples contaminated with different concentrations of *E.C.*5 (total OD_{600} = 0, 0.003, 0.006, 0.012, 0.025, 0.050, and 0.100); ellipsoids represent confidence intervals (±1 SD) for each analyte. Systematic shifts in the discriminant scores and clearer separation of clusters were observed at $OD_{600} \geq 0.050$. (B) Corresponding confusion matrix; while classification performance decreased in the lower concentration range ($OD_{600} \leq 0.025$), stable discrimination was achieved at $OD_{600} \geq 0.050$.

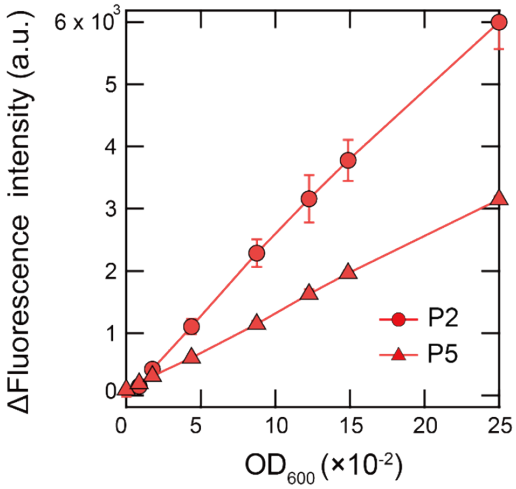


Fig. S11. Characterization of representative Dnc-polymers. Binding isotherms for **P2** and **P5** (20 μg mL^{-1}) upon addition of *A.B.* in 20 mM MOPS buffer (pH = 7.0); $\lambda_{ex}/\lambda_{em}$ = 330 nm/480 nm; values shown represent mean values ± 1 SE from three independent experiments.

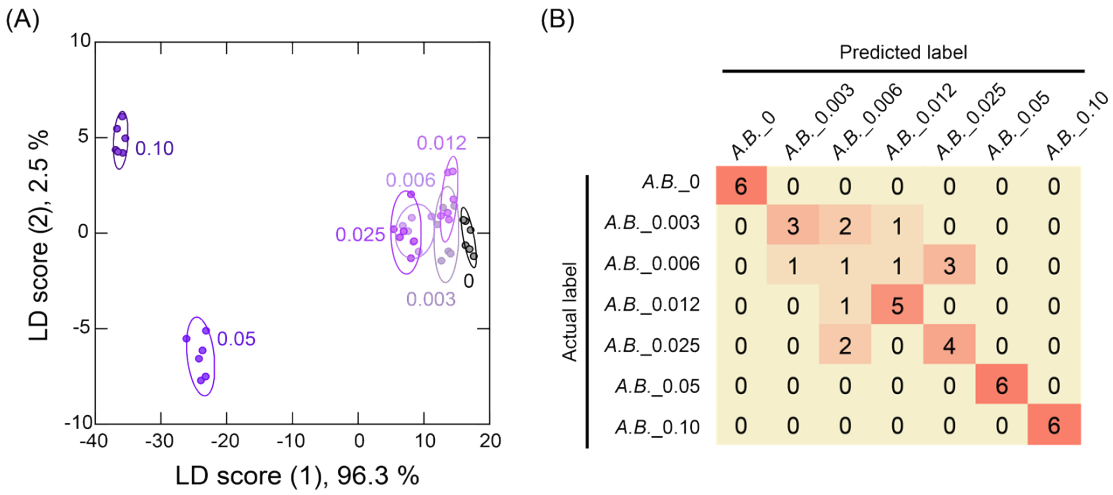


Fig. S12. (A) LDA score plots for milk samples contaminated with different concentrations of *A.B.* (total OD_{600} = 0, 0.003, 0.006, 0.012, 0.025, 0.050, and 0.100); ellipsoids represent confidence intervals (±1 SD) for each analyte. Systematic shifts in the discriminant scores and clearer separation of clusters were observed at $OD_{600} \geq 0.025$. (B) Corresponding confusion matrix. While classification performance decreased in the lower concentration range ($OD_{600} \leq 0.025$), stable discrimination was achieved at $OD_{600} \geq 0.050$.

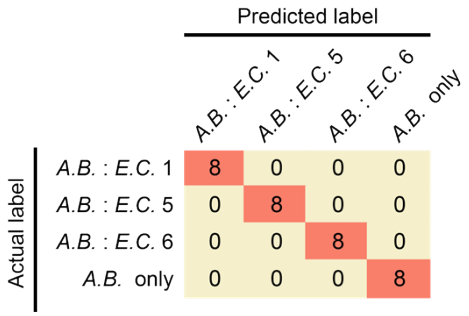


Fig. S13. Confusion matrix for the LOO cross-validation corresponding to Fig. 5A. The overall classification accuracy was 100% (24/24), with a 95% confidence interval of 86.2-100%, calculated as a Wilson binomial proportion interval.

Table. S3. Comparison of representative methods for bacterial detection and discrimination in milk.

Method	Target organism	Typical working range†	Assay time (incl. pretreatment)	Identification level	Key features / notes	Ref.
qPCR	*Foodborne bacteria*	~10^1–10^3 CFU/mL	hours	Gene-targeted (species-specific); strain-level resolution possible with advanced genomic methods	Requires DNA extraction; often requires pre-enrichment	2,3
Sensor array	*Pathogenic E. coli*	Not directly defined	≥24–48 h (culture-dependent)	Strain-level	Requires culture isolation prior to sensing; plasmonic nanomaterials	4
Sensor array	*Psychrophilic bacteria*	~10^4–10^6 CFU/mL (tested initial concentrations)	≥6 h (incubation-dependent)	Species-level; mixture discrimination	Requires incubation; plasmonic nanomaterials	5
CLSM-based imaging array	*Foodborne bacteria*	~10^8 CFU/mL	<1 h	Species-level; mixture discrimination	No cultivation; requires filtration and imaging-based analysis; CLSM combined with carbon dots	6
Sensor array	*Foodborne bacteria*	OD_{600} = 0.2 after 24 h pre-enrichment (initial ~10^4 CFU/mL)	≥24 h (pre-enrichment) + ≤14 h sensing	Species-level	Requires incubation, pre-enrichment, and buffer exchange; ssDNA–nanomaterial competitive desorption	7
This work	*E. coli* / *A. baumannii*	~10^7 CFU/mL	~2 h	Strain-level	No cultivation; requires buffer exchange (milk component removal); fluorescent polymers	-

Abbreviations: CLSM, confocal laser scanning microscopy.

† "Typical working range" refers to the bacterial concentration reported by each study, which may correspond to the concentration applied to the sensing step or to the initial concentration prior to incubation, depending on the experimental design.

Dataset S1 (separate file). Data-set matrix of fluorescence response patterns of seven different *E. coli* strains (OD_{600} = 0.1).

Dataset S2 (separate file). Data-set matrix of fluorescence response patterns of milk samples contaminated with seven different *E. coli* strains (OD_{600} =0.1).

Dataset S3 (separate file). Data-set matrix of fluorescence response patterns of milk samples contaminated with mixtures of *E.C.*1 and/or *E.C.*5 (total OD_{600} = 0.08, 0.16, 0.24).

Dataset S4 (separate file). Data-set matrix of fluorescence response patterns of milk samples containing a mixture of *A. B.* (OD_{600} = 0.1) and/or three individual *E. coli* strains (OD_{600} = 0.1).

Dataset S5 (separate file). Data-set matrix of fluorescence response patterns of time-course monitoring of milk spoilage caused by *A.B.* contamination.

Dataset S6 (separate file). Data-set matrix of fluorescence response patterns of milk samples spiked with three microbial strains (*E.C.*1, *E.C.*5, and *A. B.*; OD_{600} = 0.1) in across five different milk matrices.

References

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