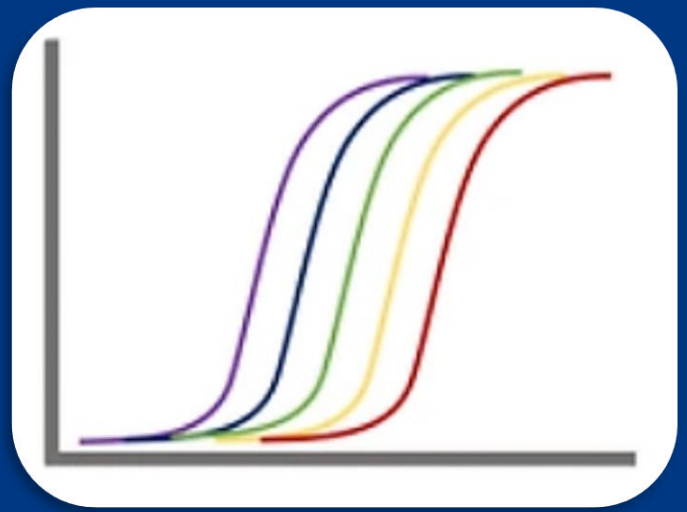
Quantitative / Real-Time PCR

qPCR for the Determination of Probiotic Potency



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Outline

- Overview of Quantitative/Real-Time PCR (qPCR)
- Benefits and drawbacks to the technology
- Differences between Digital Droplet (dd)PCR and qPCR
- Case studies using qPCR

Overview of qPCR

History

- Evolution of classic Polymerase Chain Reaction (PCR) developed in the 1980's – quantitative or real-time PCR utilizes primers/probes or dyes in conjunction with PCR reactions to detect product formation at the end of each PCR cycle instead of at the end of all cycles.
- Ability to determine the number of copies of DNA molecules present before PCR by monitoring the progress of the PCR reaction as it occurs (in real time)
- Used in many different applications

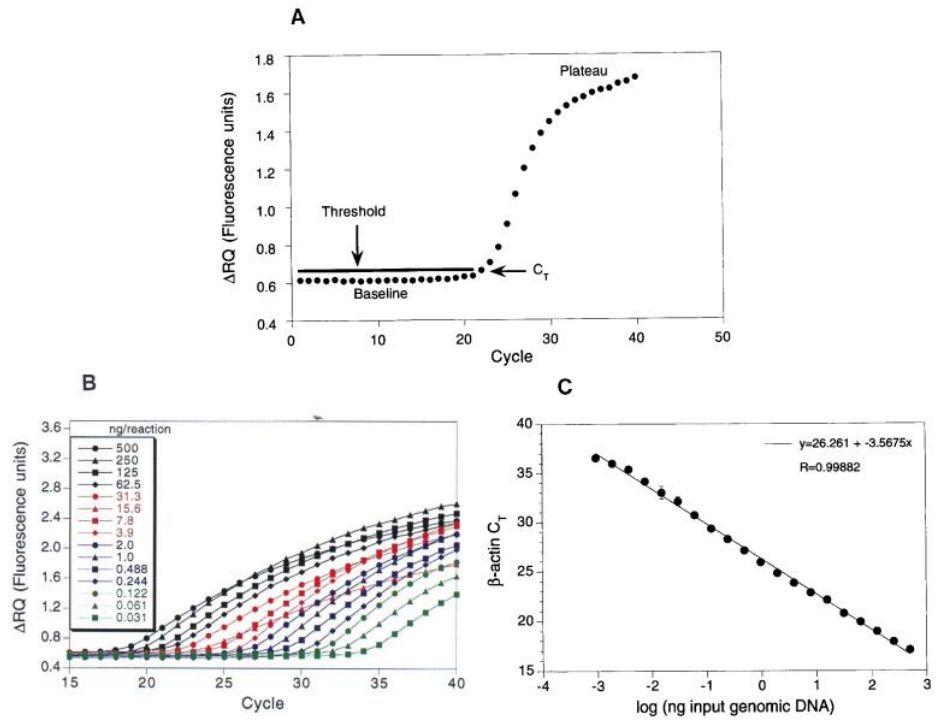


Figure 1 PCR product detection in real time. (*A*) The Model 7700 software will construct amplification plots from the extension phase fluorescent emission data collected during the PCR amplification. The standard deviation is determined from the data points collected from the base line of the amplification plot. C_T values are calculated by determining the point at which the fluorescence exceeds a threshold limit (usually 10 times the standard deviation of the base line). (*B*) Overlay of amplification plots of serially (1:2) diluted human genomic DNA samples amplified with β-actin primers. (*C*) Input DNA concentration of the samples plotted versus C_T. All points represent the mean of triplicate PCR amplifications, and error bars are shown (but not always visible).

Real time quantitative PCR.

C A Heid, J Stevens, K J Livak, et al.

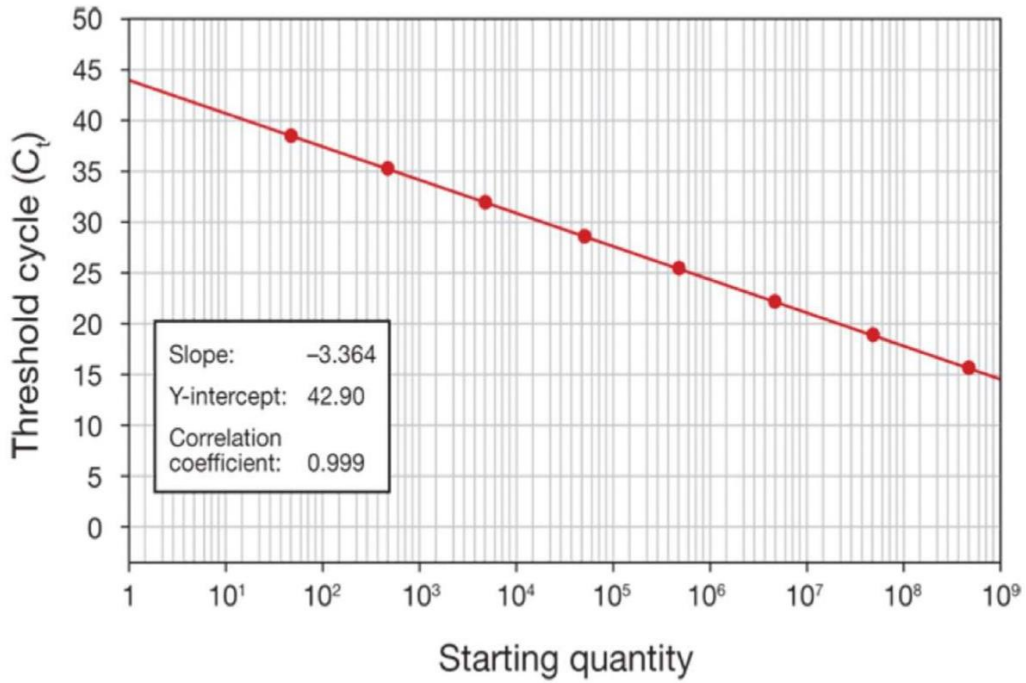
Genome Res. 1996 6: 986-994

qPCR Overview

- qPCR reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than the amount of target accumulated after a fixed number of cycles.

- The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

- Able to obtain absolute quantitation of an unknown sample by comparing to a previously determined standard curve created using nucleic acid concentrations quantified by independent means (A_{260} measurement converted to number of copies using the molecular weight of the DNA)



Figures from Fisher Scientific qPCR Handbook

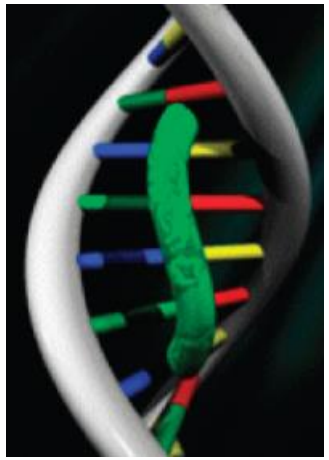
$y = m(\log x) + b$

where $y = C_t$

m = slope from best-fit line
b = y-intercept
x = quantity

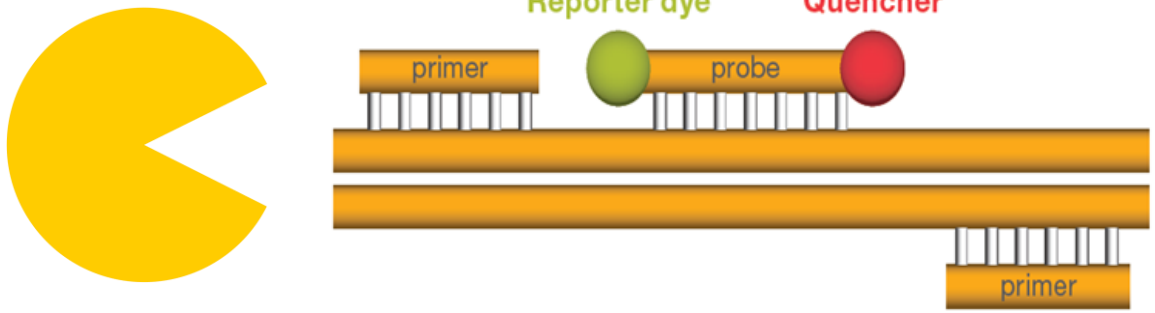
qPCR Overview

- Two types of detection are commonly used



  - SYBR Green dyes
    - Dye binds to DNA minor groove
    - More fluorescence when bound vs. unbound
    - Low specificity, so post-analysis melting curves are frequently used

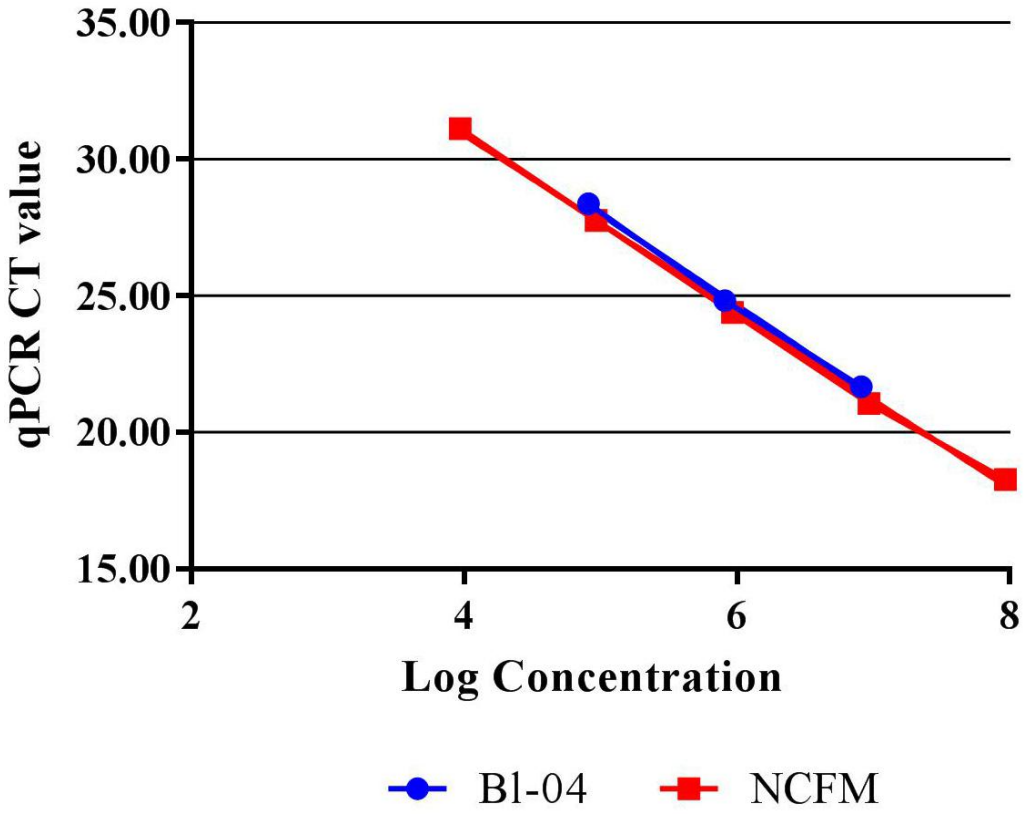
  - TaqMan probes
    - Sequence specific probe binds between two PCR primers
    - 5’ exonuclease activity of the polymerase “chews” off Reporter dye, releasing it from the Quencher and increasing fluorescence



Figures from Fisher Scientific qPCR Handbook

qPCR for Probiotic Enumeration

- Ability to perform absolute quantitation to the strain-level (ng DNA/g or ml)
- Single nucleotide polymorphisms (SNP) analysis used to create strain-specific primers and probes to use in qPCR reactions.
- Creating standard curves between Ct values and logarithmic DNA concentration or between Ct values and logarithmic cell density required.
- Target strain spiked in finished product matrix that includes other probiotic strains and standard curve established to measure resistance of the assay to interference from other strains and substrates
- Assayed by diluting sample (i.e. 10^4 to 10^8 cells) and extracting nucleic acid from the range of dilutions, and PCR reactions performed. Use Ct values to calculate the log(cell density) from the standard curve and perform equation to obtain representative CFU/g value.



Hansen SJZ, Morovic W, DeMeules M, Stahl B and Sindelar CW (2018) Absolute Enumeration of Probiotic Strains Lactobacillus acidophilus NCFM® and Bifidobacterium animalis subsp. lactis Bl-04® via Chip-Based Digital PCR. *Front. Microbiol.* 9:704. doi: 10.3389/fmicb.2018.00704

Benefits and Drawbacks of qPCR

Key Benefits

Strain-specific (inability for this specificity with plate counts)

Short time to results (hours versus days)

Higher accuracy, more precision compared to plate counts

Increase dynamic range of detection

Potential for high-throughput once curves created

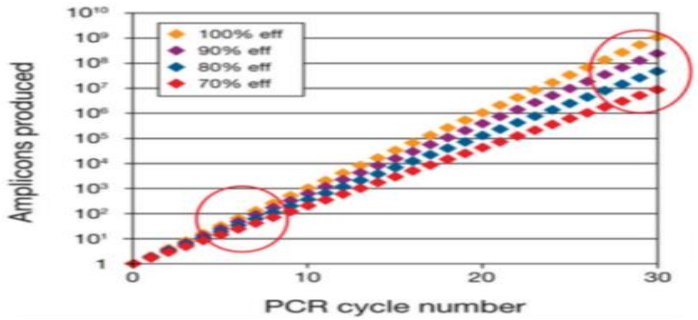
Can directly correlate to colony forming units

Drawbacks

Can’t distinguish between live and dead cells

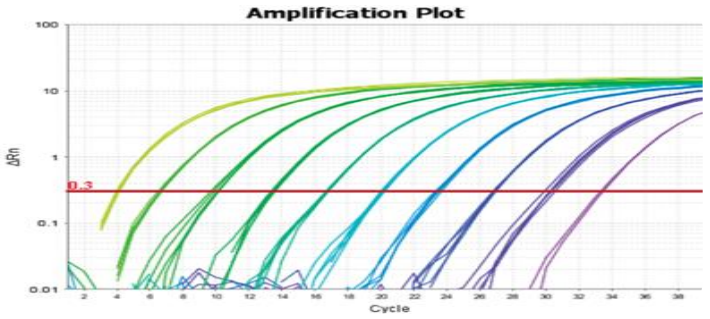
(Use dye to block amplification in dead cells)

Not a one-size-fits-all approach for probiotic enumeration



Reaction efficiency can affect quantification

(All differences in reaction efficiencies between targets, wells, or matrices will be exponentially amplified)



Figures from Fisher Scientific qPCR handbook

Standard curves needed

In order for absolute quantification to occur, samples must be compared to a known standard dilution series

Standard curves need to be from similar target organism and matrix

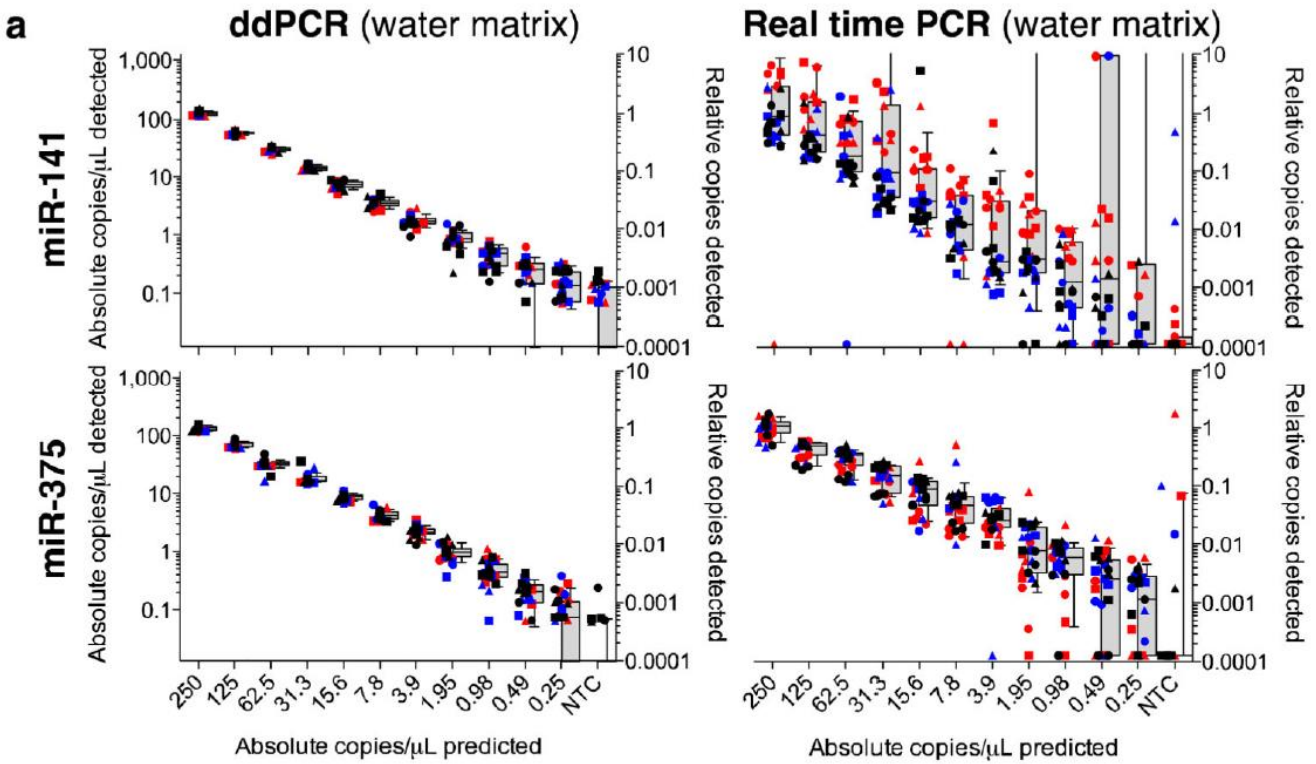
Differences between dPCR and qPCR

qPCR vs dPCR Comparison Overview

	quantitative PCR	digital PCR
Output	C_t or ΔC_t	Copies per µL
Throughput	96+	8-96
Multiplex	Up to 6 signals	Up to 2 signals
Standard curves needed	yes	no
Cost	$	$$
Results affected by PCR efficiency	yes	no

qPCR vs dPCR Precision Comparison

- **qPCR has variable precision and accuracy depending on the quality of the standard curves**
  - **Rel. SD of <25% are generally accepted**
- **dPCR advertises +/- 10% at 95% confidence interval**
  - **Others report lower levels on pure organisms depending on the type of dPCR**
    - **Chip-based dPCR shows ~3-5% rel. SD**
    - **Droplet-based dPCR shows 1.5-3% rel. SD**



Hindson, C., Chevillet, J., Briggs, H. *et al.* Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods* **10**, 1003–1005 (2013). https://doi.org/10.1038/nmeth.2633

Case Studies

Case Study 1

AEM
Journals.ASM.org

Quantitative Detection of Viable *Bifidobacterium bifidum* BF-1 Cells in Human Feces by Using Propidium Monoazide and Strain-Specific Primers

Junji Fujimoto, Koichi Watanabe
Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo, Japan

TABLE 3 Number of BF-1 cells in the feces of 12 volunteers who ingested a fermented milk product containing BF-1 for 28 days

	No. of BF-1 cells (log cells/g feces or log CFU/g feces [wet weight])					
	Quantified before ingestion[a]			Quantified after ingestion		
	qPCR[b]			qPCR		
Subject	Without PMA	With PMA	Culture	Without PMA	With PMA	Culture
a	<5.3	<5.3	<2	6.8	5.5	4.9
b	<5.3	<5.3	<2	8.1	6.2	3.3
c	<5.3	<5.3	<2	8.0	6.5	5.1
d	<5.3	<5.3	<2	8.0	6.6	6.6
e	<5.3	<5.3	<2	8.2	5.9	4.8
f	<5.3	<5.3	<2	8.5	6.6	5.3
g	<5.3	<5.3	<2	7.8	6.4	6.1
h	<5.3	<5.3	<2	8.2	5.7	2.8
i	<5.3	<5.3	<2	6.7	6.0	2.0
j	<5.3	<5.3	<2	7.6	5.9	3.7
k	<5.3	<5.3	<2	7.7	6.9	6.5
l	<5.3	<5.3	<2	5.9	5.8	2.9

[a] The lower limits of detection of qPCR and the culture method were $10^{5.3}$ cells/g feces (wet weight) and $10^{2.0}$ CFU/g feces, respectively.
[b] PMA, propidium monoazide; qPCR, real-time quantitative PCR.

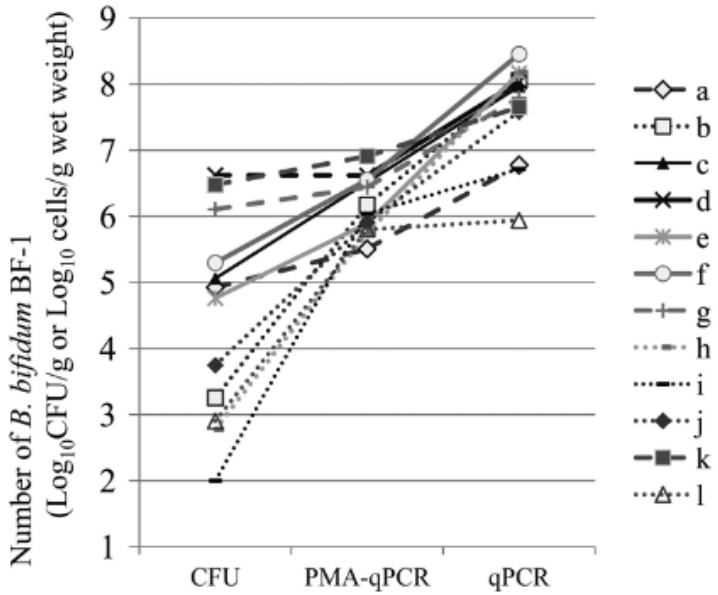


FIG 5 Number of BF-1 cells in the feces of 12 volunteers who ingested a fermented milk product for 28 days. The dashed line represents the volunteer whose number of viable BF-1 cells determined by the PMA-qPCR was 10 times less than that determined by the T-EMSM selective agar-based culture. The dotted line represents the volunteer whose number of BF-1 cells determined by PMA-qPCR was 100 times higher ($P < 0.01$) than that determined by the T-EMSM selective agar-based culture.

Case Study 2

frontiers in MICROBIOLOGY
ORIGINAL RESEARCH ARTICLE
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Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification

*Émilie Desfossés-Foucault[1], Véronique Dussault-Lepage[1], Clémentine Le Boucher[1,2], Patricia Savard[1], Gisèle LaPointe[1] and Denis Roy[1]**

[1] Département des Sciences des aliments et de nutrition, Institut des nutraceutiques et des aliments fonctionnels, Université Laval, Quebec, QC, Canada
[2] École Supérieure d'Agriculture (Groupe ESA), Angers, France

Table 5 | Quantification (log cfu/g) *of Lactococcus* sp. in all cheese samples during cheesemaking and ripening using culture media and PMA-qPCR.

Method	Cheese sample[†]	Cheesemaking steps					Ripening time (days)[††]					
		Inoculation	Cooking	Cheddaring	Salting	Pressing	30	60	90	120	150	180
Culture medium	CTL[1]	$7.4 \pm 0.1^{c*}$	8.6 ± 0.05^{ab}	9.3 ± 0.1^{a}	9.1 ± 0.1^{a}	8.7 ± 0.2^{a}	8.8 ± 0.1^{ab}	8.5 ± 0.2^{b}	7.6 ± 0.2^{cd}	7.5 ± 0.1^{cde}	7.1 ± 0.2^{de}	6.8 ± 0.2^{e}
	RO052[h]	7.5 ± 0.04^{c}	8.6 ± 0.1^{ab}	9.2 ± 0.03^{a}	9.2 ± 0.2^{a}	9.0 ± 0.1^{a}	8.0 ± 0.2^{ab}	7.4 ± 0.0^{b}	5.6 ± 0.3^{cd}	5.2 ± 0.3^{cde}	5.4 ± 0.6^{de}	4.8 ± 0.3^{e}
	RO011[i]	7.4 ± 0.r	8.6 ± 0.1^{ab}	9.2 ± 0.04^{a}	9.2 ± 0.2^{a}	9.1 ± 0.1^{a}	8.8 ± 0.1^{ab}	8.3 ± 0.2^{b}	7.5 ± 0.1^{cd}	7.1 ± 0.3^{cde}	6.9 ± 0.2^{de}	6.5 ± 0.2^{e}
	BB-12[1]	7.4 ± 0.03^{c}	8.6 ± 0.1^{ab}	9.1 ± 0.1^{a}	9.2 ± 0.2^{a}	9.1 ± 0.1^{a}	8.8 ± 0.02^{ab}	8.4 ± 0.2^{b}	8.1 ± 0.3^{cd}	7.8 ± 0.2^{cde}	7.4 ± 0.2^{de}	7.5 ± 0.4^{e}
	MC0[k]	9.0	9.9 ± 0.4^{z}	10.4 ± 0.6^{z}	10.1 ± 0.5^{z}	$10.1 \pm 0.0.4^{yz}$	9.8 ± 0.3^{wx}	9.6 ± 0.2^{vw}	8.6 ± 0.1^{uv}	8.6 ± 0.3^{uv}	NA**	NA
	MC1[1]	8.7 ± 0.3^{xy}	10.5 ± 0.1^{z}	10.7 ± 0.2^{z}	10.5 ± 0.02^{z}	10.0 ± 0.1^{yz}	6.9 ± 0.4^{wx}	6.5 ± 0.4^{vw}	5.5 ± 0.6^{uv}	6.1 ± 0.3^{uv}	NA	NA
	MC2[1]	9.0 ± 0.04^{xy}	10.5	10.7 ± 0.2^{z}	10.6 ± 0.1^{z}	10.1 ± 0.3^{yz}	7.0 ± 0.1^{wx}	6.1 ± 0.2^{vw}	5.5 ± 0.2^{uv}	4.9 ± 0.3^{uv}	NA	NA
	MC3[1]	9.0 ± 0.1^{xy}	10.6	10.7 ± 0.2^{z}	10.4 ± 0.1^{z}	10.1 ± 0.2^{yz}	7.1 ± 0.1^{wx}	6.5 ± 0.3^{vw}	5.4 ± 0.1^{uv}	5.0 ± 0.2^{uv}	NA	NA
PMA-qPCR	CTL[ij]	7.4 ± 0.3^{a}	11.1 ± 1.1^{efg}	10.9 ± 0.1^{g}	10.6 ± 0.1^{fg}	10.4 ± 0.1^{efg}	10.3 ± 0.1^{ef}	9.9 ± 0.1^{de}	9.4 ± 0.2^{cd}	9.1 ± 0.1^{bc}	8.7 ± 0.2^{ab}	8.6 ± 0.3^{ab}
	RO052[h]	8.5 ± 0.6^{a}	10.1 ± 0.1^{efg}	10.8 ± 0.1^{g}	10.6 ± 0.03^{fg}	10.3 ± 0.1^{efg}	10.0 ± 0.03^{ef}	9.3 ± 0.1^{de}	8.7 ± 0.3^{cd}	8.2 ± 0.4^{bc}	7.8 ± 0.6^{ab}	7.8 ± 0.4^{ab}
	RO011[hi]	7.7 ± 0.2^{a}	10.0 ± 0.1^{efg}	10.9 ± 0.1^{g}	10.6 ± 0.1^{fg}	10.3 ± 0.1^{efg}	10.3 ± 0.1^{ef}	9.8 ± 0.2^{de}	9.1 ± 0.3^{cd}	8.7 ± 0.3^{bc}	8.4 ± 0.4^{ab}	8.1 ± 0.4^{ab}
	BB-12[j]	7.9 ± 0.2^{a}	10.2 ± 0.03^{efg}	10.9 ± 0.1^{g}	10.7 ± 0.04^{fg}	10.3 ± 0.1^{efg}	10.3 ± 0.1^{ef}	10.1 ± 0.1^{de}	9.6 ± 0.3^{cd}	9.3 ± 0.2^{bc}	9.1 ± 0.3^{ab}	8.9 ± 0.4^{ab}
	MCO[k]	9.1 ± 0.4^{v}	11.6 ± 0.2^{w}	12.2 ± 0.2^{w}	11.9 ± 0.1^{w}	11.5 ± 0.1^{w}	11.3 ± 0.3^{uv}	10.4 ± 0.2^{uv}	9.4 ± 0.2^{u}	9.4 ± 0.3^{u}	NA	NA
	MCI[l]	8.8 ± 0.2^{v}	11.8 ± 0.2^{w}	12.2 ± 0.2^{w}	11.7 ± 0.04^{w}	11.2 ± 0.1^{w}	10.0 ± 0.1^{uv}	9.3 ± 0.3^{uv}	9.0 ± 0.3^{u}	9.0 ± 0.2^{u}	NA	NA
	MC2[l]	9.5 ± 0.3^{v}	11.6 ± 0.1^{w}	12.0 ± 0.2^{w}	11.8 ± 1.0^{w}	11.7 ± 0.3^{w}	9.4 ± 0.2^{uv}	9.2 ± 0.1^{uv}	8.6 ± 0.1	8.4 ± 0.1^{u}	NA	NA
	MC3[l]	10.4 ± 0.6^{v}	11.6 ± 0.2^{w}	11.5 ± 0.6^{w}	11.6 ± 0.2^{w}	11.2 ± 0.1^{w}	9.6 ± 0.1^{uv}	9.1 ± 0.1^{uv}	8.7 ± 0.1^{u}	8.8 ± 0.3^{u}	NA	NA

[†] CTL, control for the single culture batch; BB-12, B. animalis subsp. lactis BB-12 in single culture; RO011, L. rhamnosus RO011 in single culture; RO052, L. helveticus RO052 in single culture; MCO, control cheese for the mixed culture batch; MCI, RO052+BB-12; MC2, RO052+RO011; MC3, RO052+RO011+BB-12.

[a–g] Results for single culture cheese samples not horizontally connected with the same letter indicate a significant difference between cheesemaking and ripening times (Tukey–Kramer HSD test; $P < 0.05$).

[u–z] Results for mixed culture cheese samples not horizontally connected with the same letter indicate a significant difference between cheesemaking and ripening times (Tukey–Kramer HSD test; $P < 0.05$).

[h–j] Results for single culture cheese samples not vertically connected with the same letter indicate a significant difference between samples (Tukey–Kramer HSD test; $P < 0.05$).

[k, l] Results for mixed culture cheese samples not vertically connected with the same letter indicate a significant difference between cheese samples (Tukey–Kramer HSD test; $P < 0.05$).

**All results are means followed by their standard deviation (SD).*

***NA, not analyzed for this point.*

Case Study 2

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Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification

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Table 6 | Quantification (log cfu/g) of each probiotic species in all cheese samples during cheesemaking and ripening using culture media and PMA-qPCR.

Method	Quantified species	Cheese sample[†]	Cheesemaking steps					Ripening time (days)					
			Inoculation	Cooking	Cheddaring	Salting	Pressing	30	60	90	120	150	180
Culture Media	*L. rhamnosus*	RO0II	$6.5 \pm 0.02^{a*}$	6.8 ± 0.2^{ab}	7.0 ± 0.2^{abc}	7.1 ± 0.2^{abc}	7.8 ± 0.1^{cd}	8.0 ± 0.1^{d}	7.7 ± 0.2^{cd}	7.5 ± 0.1^{bcd}	7.5 ± 0.2^{cd}	7.5 ± 0.1^{bcd}	7.5 ± 0.1^{bcd}
		MC2[k]	8.2 ± 0.04^{u}	9.1 ± 0.03^{u}	9.3 ± 0.1^{v}	9.1 ± 0.1^{v}	9.5 ± 0.1^{v}	9.3 ± 0.1^{v}	9.4 ± 0.1^{v}	9.3 ± 0.1^{v}	9.2 ± 0.2^{v}	NA**	NA
		MC3[k]	8.4 ± 0.1^{u}	8.8 ± 0.4^{u}	9.4 ± 0.02^{v}	9.2 ± 0.1^{v}	9.5 ± 0.2^{v}	9.3 ± 0.2^{v}	9.4 ± 0.1^{v}	9.4 ± 0.2^{v}	9.8 ± 0.3^{v}	NA	NA
	L. helveticus	RO052	6.9 ± 0.3^{ab}	7.1 ± 0.1^{ab}	7.6 ± 0.03^{b}	7.8 ± 0.1^{b}	8.1 ± 0.1^{b}	7.9 ± 0.2^{b}	7.8 ± 0.3^{b}	7.5 ± 0.0^{b}	7.3 ± 0.1^{b}	7.4 ± 0.2^{b}	6.7 ± 0.2^{ab}
		MCI[k]	8.5 ± 0.1^{u}	9.2 ± 0.02^{vwx}	9.5 ± 0.02^{x}	9.4 ± 0.1^{vwx}	9.6 ± 0.1^{x}	9.5 ± 0.1^{vwx}	9.4 ± 0.04^{wx}	9.2 ± 0.2^{vwx}	8.8 ± 0.2^{uv}	NA	NA
		MC2[k]	8.5 ± 0.1^{u}	9.3 ± 0.1^{vwx}	9.5 ± 0.05^{x}	9.4 ± 0.03^{vwx}	9.6 ± 0.1^{x}	9.4 ± 0.1^{vwx}	9.7 ± 0.1^{wx}	9.3 ± 0.1^{vwx}	8.9 ± 0.2^{uv}	NA	NA
		MC3[k]	8.7 ± 0.1^{u}	9.3 ± 0.1^{vwx}	9.6 ± 0.1^{x}	9.3 ± 0.1^{vwx}	9.9 ± 0.3^{x}	9.5 ± 0.01^{vwx}	9.4 ± 0.1^{wx}	9.5 ± 0.2^{vwx}	9.0 ± 0.1^{uv}	NA	NA
	B. animalis subsp. *lactis*	BB-12	7.1 ± 0.1^{a}	7.5 ± 0.1^{b}	8.0 ± 0.1^{c}	8.2 ± 0.03^{c}	8.3 ± 0.1^{c}	8.3 ± 0.03^{c}	8.2 ± 0.1^{c}	8.0 ± 0.1^{bc}	8.0 ± 0.1^{bc}	8.0 ± 0.1^{c}	7.9 ± 0.1^{bc}
		MCI[k]	8.7 ± 0.02^{u}	9.4 ± 0.02^{vwx}	9.8 ± 0.1^{wx}	10.1 ± 0.3^{x}	9.7 ± 0.2^{wx}	9.5 ± 0.2^{vw}	9.5 ± 0.1^{vw}	9.0 ± 0.3^{uv}	8.9 ± 0.3^{uv}	NA	NA
		MC3[k]	8.7 ± 0.03^{u}	9.5 ± 0.1^{vwx}	9.8 ± 0.1^{wx}	10.0 ± 0.3^{x}	9.8 ± 0.2^{wx}	9.3 ± 0.1^{vw}	9.4 ± 0.04^{vw}	9.0 ± 0.1^{uv}	8.9 ± 0.2^{uv}	NA	NA
PMA-qPCR	*L. rhamnosus*	RO0II	5.7 ± 0.2^{a}	7.4 ± 0.02^{bc}	7.9 ± 0.1^{cd}	7.8 ± 0.1^{cd}	8.1 ± 0.1^{d}	7.1 ± 0.2^{b}	7.4 ± 0.1^{bc}	7.1 ± 0.2^{b}	7.3 ± 0.1^{bc}	7.2 ± 0.02^{b}	7.4 ± 0.1^{bc}
		MC2[k]	6.4 ± 0.4^{u}	8.1 ± 0.1^{v}	8.2 ± 0.2^{vw}	8.3 ± 0.2^{vw}	8.6 ± 0.2^{w}	8.6 ± 0.1^{w}	8.5 ± 0.1^{w}	8.5 ± 0.1^{w}	8.6 ± 0.1^{w}	NA	NA
		MC3[k]	6.7 ± 0.2^{u}	7.8 ± 0.1^{v}	8.5 ± 0.1^{vw}	8.4 ± 0.2^{vw}	8.6 ± 0.1^{w}	8.7 ± 0.1^{w}	8.5 ± 0.1^{w}	8.5 ± 0.1^{w}	8.5 ± 0.1^{w}	NA	NA
	L. helveticus	RO052	5.2 ± 0.2^{a}	7.1 ± 0.2^{b}	7.8 ± 0.2^{vw}	7.9 ± 0.1^{c}	8.3 ± 0.1^{cd}	8.8 ± 0.03^{d}	8.7 ± 0.1^{d}	8.5 ± 0.1^{d}	8.7 ± 0.1^{d}	8.6 ± 0.01^{d}	8.5 ± 0.1^{d}
		MC1[k]	6.9 ± 0.3^{u}	9.3 ± 0.1^{v}	9.5 ± 0.2^{vw}	9.5 ± 0.2^{vwx}	9.8 ± 0.1^{wx}	9.8 ± 0.1^{wx}	9.9 ± 0.1^{wx}	9.5 ± 0.1^{wx}	9.9 ± 0.1^{x}	NA	NA
		MC2[k]	7.8 ± 0.3^{u}	9.3 ± 0.1^{v}	9.5 ± 0.2^{vw}	9.6 ± 0.2^{vwx}	10.0 ± 0.1^{wx}	9.7 ± 0.03^{wx}	9.9 ± 0.1^{wx}	9.9 ± 0.1^{wx}	10.0 ± 0.1^{x}	NA	NA
		MC3[k]	7.7 ± 0.3^{u}	9.1 ± 0.1^{v}	9.6 ± 0.1^{vw}	9.6 ± 0.1^{c}	9.8 ± 0.04^{wx}	9.7 ± 0.1^{wx}	9.8 ± 0.1^{wx}	9.9 ± 0.1^{wx}	10.0 ± 0.1^{x}	NA	NA
	B. animalis subsp. *lactis*	BB-12	6.0 ± 0.2^{a}	8.2 ± 0.1^{b}	8.9 ± 0.1^{c}	9.0 ± 0.04^{c}	9.1 ± 0.1^{c}	9.1 ± 0.1^{c}	9.2 ± 0.1^{c}	8.8 ± 0.2^{bc}	8.6 ± 0.1^{bc}	8.8 ± 0.1^{c}	8.7 ± 0.2^{bc}
		MC1[k]	7.5 ± 0.02^{u}	10.3 ± 0.1^{vwx}	10.6 ± 0.1^{x}	10.5 ± 0.04^{x}	10.5 ± 0.1^{x}	10.5 ± 0.1^{vwx}	10.3 ± 0.03^{vwx}	9.9 ± 0.2^{v}	10.0 ± 0.3^{vw}	NA	NA
		MC3[k]	8.8 ± 0.2^{u}	10.1 ± 0.1^{vwx}	10.8 ± 0.1^{x}	10.6 ± 0.05^{x}	10.6 ± 0.1^{x}	10.3 ± 0.04^{vwx}	10.1 ± 0.2^{vwx}	9.7 ± 0.1^{v}	9.7 ± 0.2^{vw}	NA	NA

[†]CTL, control for the single culture batch; BB-12, B. animalis subsp. lactis BB-12 in single culture; RO011, L. rhamnosus RO011 in single culture; RO052, L. helveticus RO052 in single culture; MCO, control cheese for the mixed culture batch; MC1, RO052+BB-12. MC2, RO052+RO011; MC3, RO052+RO011+BB-12.

[a–g]Results for single culture cheese samples not horizontally connected with the same letter indicate a significant difference between cheesemaking and ripening times (Tukey–Kramer HSD test; $P < 0.05$).

[u–x]Results for mixed culture cheese samples not horizontally connected with the same letter indicate a significant difference between cheesemaking and ripening times (Tukey–Kramer HSD test; $P < 0.05$).

[h–l]Results for single culture cheese samples not vertically connected with the same letter indicate a significant difference between samples (Tukey-Kramer HSD test; $P < 0.05$).

[k]Results for mixed culture cheese samples not vertically connected with the same letter indicate a significant difference between cheese samples (Tukey–Kramer HSD test; $P < 0.05$).

**All results are means followed by their standard deviation (SD).*

***NA, not analyzed for this time point.*

Thank You

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