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List of Abbreviations and Acronyms	
AnSBR	Anaerobic sequencing batch reactor
CW	Cheese Whey
VFAs	Volatile fatty acids
HRT	Hydraulic retention time
SRT	Solid retention time
OFMSW	Organic fraction of municipal solid waste
PBBR	Packed bed biofilm reactor
CSTR	Continuous stirred tank reactor
OLR	Organic loading rate
ORP	Oxidation-reduction potential
DO	Dissolved oxygen
COD	Chemical oxygen demand
TSS	Total suspended solids
VSS	Volatile suspended solids

1 Executive Summary

This deliverable presents the results of Subtask 2.1.1, which aimed to develop and optimise an anaerobic fermentation process for the valorisation of cheese whey into volatile fatty acids (VFAs), operating under mesophilic, semi-batch conditions. Five experimental phases were carried out to investigate the influence of key parameters—pH, hydraulic retention time (HRT), solid retention time (SRT), and microbial inoculation—on VFA yield, composition, and process stability. The initial batch trial without pH control or inoculum confirmed negligible VFA production and underscored the necessity of maintaining a neutral pH and sufficient residence time to trigger acidogenesis. Subsequent semi-batch trials demonstrated that both pH regulation and inoculation with *Propionibacterium freudenreichi subsp shermanii* significantly improved VFA yields and selectivity. Peak performance was achieved under controlled pH 6.5–7.0, with HRT of 10 days and SRT of 20 days, reaching a maximum of 20.2 g/L VFAs and a butyric acid content exceeding 80%. Process intensification (HRT down to 4–6 days) was feasible with only moderate yield reductions, provided pH remained stable. Redox (ORP) and correlation analyses further highlighted pH as the most influential driver of VFA productivity. These findings support the transition to pilot-scale fermentation (500 L) with implementation of continuous pH control and nitrogen purging strategies to ensure anaerobicity. The optimised configuration will provide the fermentate to be tested in downstream PUFA production (Task 2.2), contributing to the circular valorisation of dairy residues within the ONE-EARTH project.

2 Context and Objective of Anaerobic Fermentation Trials

Anaerobic fermentation represents a promising approach for the valorisation of organic waste streams through the production of volatile fatty acids (VFAs), which are key intermediates for the synthesis of value-added products such as bioplastics, biofuels, and fine chemicals. In recent years, increasing attention has been devoted to short-chain VFAs (acetic, propionic, butyric acids) as renewable chemical building blocks with applications in the energy, chemical, and pharmaceutical sectors. Among various feedstocks, cheese whey is particularly suitable for anaerobic acidogenic fermentation. As a by-product of dairy industries, it accounts for 85–95% of the milk volume used during cheese production and retains over half of its nutrients. Its composition—rich in lactose, soluble proteins, and with a high COD (chemical oxygen demand)—makes it highly biodegradable and an ideal substrate for microbial conversion under anaerobic conditions. However, if not properly managed, cheese whey poses a significant environmental burden due to its organic load. The acidogenic fermentation of cheese whey is strongly influenced by several operational parameters, including pH, temperature, hydraulic and solid retention times (HRT and SRT), and inoculum selection. Literature evidence suggests that by modulating these factors, it is possible to selectively steer microbial metabolism toward the production of specific VFAs. For example, acidic pH (5–6) favours butyric acid production, whereas neutral pH (7) tends to support higher acetic acid yields. Retention times are also critical, as they affect both the degradation kinetics of complex substrates (e.g., lactose and lactic acid) and the stability of microbial communities.

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Within this context, the activities described in this deliverable were carried out in the framework of WP2 – Upgrading of fermentation strategies for tailored VFA production, and specifically under Task 2.1 – VFA production from cheese whey. The core objective of Subtask 2.1.1 was to design, implement, and optimise a laboratory-scale anaerobic fermentation process operated under mesophilic conditions, aiming to maximise VFA yield and recovery from real cheese whey provided by the industrial partner Mambelli.

The experimental strategy was structured in three progressive phases, each addressing a specific process parameter: first assessing the baseline fermentability of cheese whey in batch mode, then exploring the effect of pH control and inoculum addition in semi-batch trials and finally investigating reduced retention times under optimised conditions. The VFAs produced in the process were intended to serve as substrates for PUFA production, in line with the integrated cascade valorisation approach outlined in the project proposal (Task 2.2).

The following sections report in detail the methodology adopted, the process configurations tested, and the results obtained, placing them in direct relation to the objectives of WP2 and highlighting whether expected outcomes were achieved and what steps are foreseen for further optimisation and scale-up.

3 Aim of the Study

This study was carried out within Task 2.1 – VFA production from cheese whey, specifically addressing Subtask 2.1.1, which aims to establish and optimise an anaerobic acidogenic fermentation process at TRL 3 using CW collected from the "Squacquerone" cheese production facility Mambelli, located in Bertinoro (Emilia-Romagna, Italy).

The specific objectives of the study were:

1. To set up a lab-scale fermentation system (1L) operated in mesophilic conditions (37 °C) and fed with CW following a semi-batch strategy.
2. To optimise key process parameters for acidogenesis, including pH, hydraulic retention time (HRT), solid retention time (SRT), and the use of inoculated versus non-inoculated conditions.
3. To compare batch and semi-batch modes to assess which configuration results in higher VFA production and process stability.
4. To characterise the fermentation effluents in terms of VFA concentration and composition, in order to inform process adjustments.
5. To evaluate post-treatment strategies for the recovery and concentration of VFAs from the effluent.
6. To deliver VFA-rich effluents to partners in Task 2.2, enabling downstream applications focused on PUFA production.

4 Process Parameters and Laboratory-scale Configurations from scientific literature review

The primary operational parameters for the anaerobic fermentation process, as identified in the literature to investigate their impact on VFA yields, are summarised in Table 1. The whole and comprehensive literature review underpinning the definition of the various test conditions was presented and summarised in Milestone 2.

Table 1. Key operational parameters and reactor setup for anaerobic fermentation, as reported in the literature.

Reactor configuration	Process mode	Working volume (L)	Temperature (°C)	pH	Cheese whey type	Inoculum type	SRT (d)	HRT (d)	OLR (g COD/L.d)	References
PBBR	Continuous	0.8	37	6-7	Powder	Anaerobic fermentation consortium	-	4, 6		Domingos et al. [1]
AnSBR	Semi-batch	2	30	5, 5.5, 6	Liquid	AD sludge	4, 6, 10	2	3-12	Calero et al. [2]
CSTR	Semi-batch	2	35	No control	Liquid	Anaerobic sludge	-	1, 2, 4	-	Luongo et al. [3]
CSTR	Batch	2	37	No control, 5.5, 9	Liquid	Sewage sludge	-	10, 20	-	Iglesias et al. [4]
AnSBR	Semi-batch	2	30	5	Liquid	Mixed culture from AF reactor	5, 10, 15	1, 2, 3	6	Lagoa Costa et al. [5]
AnSBR	Batch/ Semi-batch	1	37	No control, 6, 7	Liquid	No inoculum/ commercial *propionic strains*	35[1], 20[2], 12[3,4]	10[1,2], 6[3] & 4[4]		ONE EARTH activity

[1]Phase 2; [2]Phase 3; [3]Phase 4; [4]Phase 5

5 Materials and Methods

This section describes the experimental setup and procedures adopted for the development of the anaerobic fermentation process of cheese whey (CW) for the production and recovery of volatile fatty acids (VFAs), in line with the objectives defined under Task 2.1. The section is structured as follows:

- Section 5.1: Materials used in the study, including the origin and types of cheese whey and reactor device.

- Section 5.2: Description of the experimental approach, structured into five sequential phases aligned with specific objectives.
- Section 5.3: Analytical procedures and monitoring strategies applied to evaluate process performance.
- Section 5.4: Post-treatment methods for VFA recovery.
- Section 5.5: Quality and reproducibility aspects.

5.1 Experimental Approach: Description of device and general set-up

Anaerobic fermentation trials were performed in various phases with the main aim of continuous process optimisation for VFA production yield, each focusing on specific operational parameters to enhance the process performance and conditions.

Cheese whey was collected from a local dairy facility in Bertinoro (Emilia-Romagna, Italy), specialising in the production of Squacquerone and Ricotta cheese. The whey was delivered fresh and stored at 4 °C before use. While both Squacquerone and Ricotta whey were utilized only in Phase 1, subsequent trials were conducted only with Squacquerone whey for the purposes discussed in section (5.2.1).

All experiments were conducted using a bench-scale anaerobic fermentation system consisting of two glass vessels (1 L working volume each). The vessels were equipped with:

- A mechanical stirring system.
- An integrated Peltier system (10–50 °C) for automated temperature control.
- External multi-meters (Hach Lange HQ2100) for real-time pH and ORP (oxidation-reduction potential) monitoring.

pH was adjusted in Phases 2-5 using 10 M NaOH and 2 M H_2SO_4. Besides, during batch trials (Phase 1), nitrogen was supplied to remove any dissolved oxygen in cheese whey's and to ensure optimal oxidation-reduction potential (ORP) for acidogens.

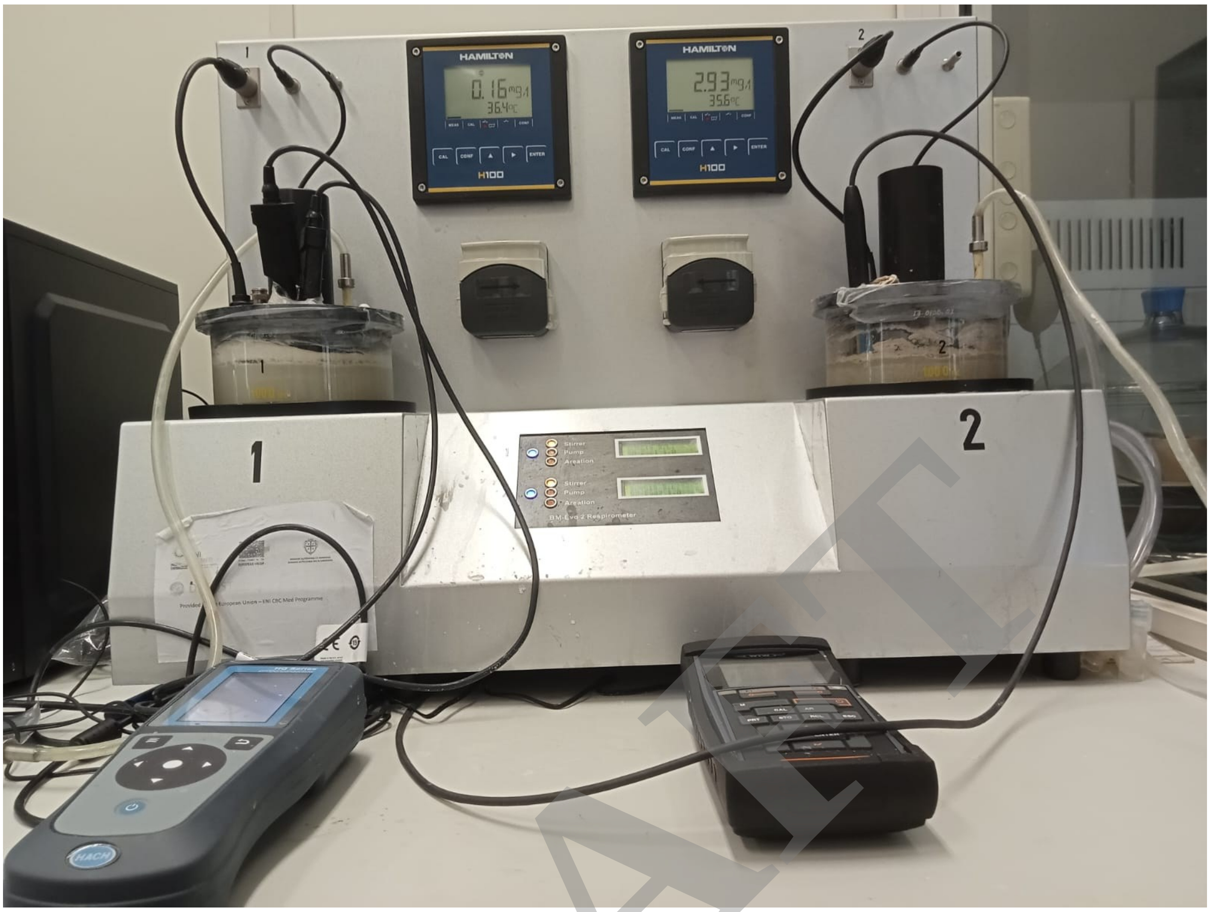

Figure 1. Laboratory-scale anaerobic fermentation setup.

5.2 Experimental Approach: Process Phases and Strategies

This subsection outlines the sequential experimental strategy adopted to develop and optimise the anaerobic fermentation process for VFA production from cheese whey, in line with the objectives of Subtask 2.1.1. The trials were structured in five main phases (Table 2), each designed to investigate specific operational modes and variables such as batch vs semi-batch mode, SRT, HRT, pH, and inoculum addition:

- **Phase 1**: Batch trials without inoculum or pH control, to assess native microbial activity and optimal HRT.
- **Phase 2**: Semi-batch trials with and without (control) inoculum, under controlled pH (6).
- **Phase 3**: Semi-batch trials with and without (control) inoculum at pH 7, with reduced SRT to improve process efficiency.
- **Phase 4:** Semi-batch trials with and without (control) inoculum at pH 7, with reduced SRT (12 days) and HRT (6 days) to investigate shorter SRT and HRT influence on process efficiency.
- **Phase 5:** Semi-batch trials with and without (control) inoculum at pH 7, with reduced SRT (12 days) and HRT (4 days) to investigate shorter HRT influence on process efficiency.

Table 2. Laboratory-scale fermentation process phases and variables tested

Phase n°	Process mode	Main variables tested				Trials duration (days)
		Inoculum	pH	HRT (d)	SRT (d)	
Phase 1	Batch	no	no	2 – 5 - 10	-	2- 5 - 10
Phase 2	Semi-batch	yes	**6**	10	36	36
Phase 3	Semi-batch	yes	**7**	10	20	26
Phase 4	Semi-batch	yes	7	**6**	12	16
Phase 5	Semi-batch	yes	7	**4**	12	12

The results obtained from each phase informed the design and operational choices of the subsequent one, progressively refining process conditions to enhance acidogenic performance.

5.2.1 Phase 1: Batch scale trials

Phase 1 was carried out in batch mode. 3 different hydraulic retention times, namely, 2, 5 and 10 days, were evaluated to understand process performance and VFA yield. These trials were performed without inoculum addition (commercial bacterium strains or anaerobic digestor sludge, etc) or pH control. Specific objectives were to evaluate the VFA production potential of the native microbial consortium in cheese whey at natural pH, and to identify optimal HRT. Two types of cheese whey, namely, Squacquerone and Ricotta cheese whey, were utilised. The main process parameters are given in Table 3.

Table 3. Operational parameters for phase 1.

Parameter	Unit	Value
Operation mode	-	Batch
Temperature	°C	37
HRTs	days	2, 5 and 10
pH	-	No control
Nitrogen supply	seconds	Intermittent supply

5.2.2 Phase 2: Semi-batch scale trials

Following the observation and findings from phase 1, the next fermentation phase 2 was performed. Specifically, the operation mode was switched to semi-batch as fermentation effluents from all previous batch trials were still rich in lactose as well as lactic acid, which potentially implied the need for longer residence times for biomass to acclimatise and effectively transform both lactose and lactic acid into VFAs. In phase 2, two parallel trials were carried out, one with commercial *Propionibacterium strains* (*Propionibacterium freudenreichi subsp shermanii)*; prepared in saline solution) and the other as a control test (without inoculum addition). These trials focused exclusively on Squacquerone cheese whey for following key reasons: (1) it is the main type of whey produced by the supplier, making it the most practical and scalable option for further investigation (2) it yielded higher acetic acid concentrations than Ricotta whey, as discussed in Section 6.2, (3) it still contained a significant amount of lactic acid, which can serve as a substrate for VFA-producing bacteria, and (4) time constraints limited the feasibility of testing additional whey types.

Reactors were operated in Phase 2 for 36 days (SRT) with 10 days hydraulic retention time (HRT) and a pH of 6 to investigate the influence of pH on providing favourable conditions for VFA-producing bacteria. pH was manually adjusted every day after the collection of samples. To avoid biomass washout, the mixer was stopped for 30 minutes to settle the biomass, and samples were collected for analysis. During this operation, a volume of 100mL was collected and replaced by the same amount of fresh cheese whey. The main process parameters are presented in Table 4.

Table 4. Operational parameters for phase 2.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	35
HRT	days	10
pH	-	6
pH control	-	Manual
pH buffer	-	10M NaOH or 2M H_2SO_4
Feed volume	daily	100 mL
VFA effluent collected	daily	100 mL

5.2.3 Phase 3: Semi-batch scale trials

Phase 3 was carried out in response to observations from Phase 2, making two key changes: reducing the solids retention time (SRT) to 20 days and evaluating process performance for optimising VFA yield at pH 7. Biomass was purged every other day without allowing it to settle, to avoid the buildup of dead or inactive biomass that could hinder process efficiency. Reactors were operated for 26 days (SRT) under the same HRT of 10 days. The main process parameters are summarised in Table 5.

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Table 5. Operational parameters for phase 3.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	20
HRT	days	10
pH	-	7
pH adjustment	-	Manual
pH buffer	-	10M NaOH or 2M H_2SO_4
Feed volume	daily	100 mL
VFA effluent collected	daily	100 mL

5.2.4 Phase 4: Semi-batch scale trials

Phase 3 was carried out in response to observations from Phase 3, making two key changes: reducing the solids retention time (SRT) to 12 days and HRT to 6 days, to evaluate the influence of shorter solid and hydraulic retention times on VFA yield, while keeping pH at 7. Biomass was purged every other day without allowing it to settle, to avoid the buildup of dead or inactive biomass that could hinder process efficiency. Reactors were operated for 2 complete operation cycles (HRT), lasting 16 days. The main process parameters are summarised in Table 6.

Table 6. Operational parameters for phase 4.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	12
HRT	days	6
pH	-	7
pH adjustment	-	Manual
pH buffer	-	10M NaOH or 2M H_2SO_4
Feed volume	daily	167 mL
VFA effluent collected	daily	167 mL

5.2.5 Phase 5: Semi-batch scale trials

To further investigate the influence of shorter HRT on obtaining higher throughput with higher VFA yield, a HRT of 4 days was evaluated with SRT (12 days) and pH (7). Similar to Phase 4, reactors were operated for 2 complete operation cycles (HRT), lasting 12 days. The main process parameters are summarised in Table 7.

Table 7. Operational parameters for phase 5.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	12
HRT	days	4
pH	-	7
PH adjustment	-	Manual
pH buffer	-	10M NaOH or 2M H_2SO_4
Feed volume	daily	250 mL
VFA effluent collected	daily	250 mL

5.3 Instrumentation and Analytical Equipment

This section describes the monitoring strategy and analytical methods adopted to evaluate process performance and characterise both raw materials and fermentation effluents. It includes the parameters measured, the equipment used, and the sample treatment procedures applied throughout the experimental trials.

5.3.1 Monitoring procedures:

Basic monitoring included daily measurement of:

- pH
- ORP (oxidation-reduction potential)
- DO (dissolved oxygen)

pH and ORP were monitored using Hach Lange HQ2100 multi-meters, while DO was monitored by the reactor.

5.3.2 Analytical Parameters

Samples were withdrawn and replaced with fresh CW. Before analysis, samples were:

1. Centrifuged at 6000 rpm for 10 minutes.
2. Filtered using 0.45 µm vacuum filters.

The following parameters were analysed in raw and fermentation throughput:

- VFAs (formic, acetic, propionic, butyric acids) and lactic acid by Ion Chromatography (HPIC). Sampling frequency was adjusted according to HRT:
    - For longer HRTs (10 days): samples analysed every 3–4 days.
    - For intermediate HRT (6 days): samples analysed every 2–3 days.
    - For shorter HRT (4 days): samples analysed daily to observe fermentation dynamics.
- COD (Chemical Oxygen Demand, total and soluble).
- Ammonia (NH_4^+) concentration.
- Cation/Anion profiles.
- TSS (Total Suspended Solids) and VSS (Volatile Suspended Solids).

5.3.3 Post-treatment for VFA recovery

To recover VFAs from the fermentation liquid, literature was consulted to adopt an efficient post-treatment method. A combination of centrifugation followed by filtration was used, modifying the methodology adopted in [6]. The liquid samples were first centrifuged at 6000 rpm for 10 minutes, then vacuum filtered using 0.45 µm filters to recover the VFA-rich liquid.

5.3.4 Quality and Reproducibility Aspects

1. Starting Phase 2 and onwards, trials were conducted in parallel as discussed in 5.2.2. The objective was to evaluate the performance of the commercial *Propionibacterium* inoculum compared to the inherent microbial consortium in input cheese whey.
2. CW was stored at 4°C to facilitate the daily addition of fresh CW into reactors to allow working in semi-batch mode.
3. VFA-rich liquid samples, before the analysis, were post-treated as discussed in the section 5.3.3.
4. Analysis of soluble compounds, including COD soluble, ion strength, and Ammonia, was performed in duplicate.

6 Results and Discussion

This section provides a structured overview of the activity's results, outlining the implementation phases and challenges encountered.

6.1 Initial Setup and Cheese Whey Characterisation (Month 1–6)

A comprehensive literature review was conducted to understand the key process parameters that influence the anaerobic fermentation process, and to select process parameters for laboratory-scale trials (Milestone 2). During the same period, a sampling campaign was conducted to collect the cheese whey sample for preliminary characterisation, presented in Table 8 to Table 11.

Physicochemical analysis showed that Squacquerone whey had lower solids content than Ricotta whey (4.4 vs 14.9 g/L), but similar COD values (74 vs 76 g/L). Both matrices exhibit a slightly acidic initial pH and a good nutrient content, particularly phosphorus. Both raw whey samples were characterised for their initial VFA profiles, as presented in Table 9 and Table 11.

Table 8. Physico-Chemical Characteristics of Squacquerone CW.

Parameter	Units	Average	Std
pH	-	6.47	0.01
N-NH4	g/L	0.114	-
TSS	g/L	4.38	0.22
VSS	g/L	4.12	0.16
VSS/TSS	%	0.941	-
P-PO4	g/L	0.534	0.006
P-PO4	g/kg	0.541	0.006
COD$_{total}$	g/L	74.38	7.20
COD$_{soluble}$	g/L	55.3	0.31
Density	kg/L	1.012	-

Table 9. VFA and lactic acid concentration in raw Squacquerone whey

VFA and lactic acid concentration	Units	Value
Formic acid	mg/L	54
Acetic acid	mg/L	670
Propionic acid	mg/L	<5
Lactic acid	mg/L	9000

Table 10. Physico-Chemical Characteristics of Ricotta CW.

Parameter	Units	Average	Std
pH	-	6.28	0.01
N-NH4	mg/L	-	-
TSS	g/L	14.86	0.46
VSS	g/L	14.68	0.53
VSS/TSS	%	0.988	-
P-PO4	g/L	0.446	0.001
P-PO4	g/kg	0.451	0.001
COD_{total}	g/L	76.42	2.40
$COD_{soluble}$	g/L	52.44	0.63
Density	kg/L	1.0117	-

Table 11. VFA and lactic acid concentration in raw Ricotta whey

VFA and lactic acid concentration	Units	Value
Formic acid	mg/L	17
Acetic acid	mg/L	1200
Propionic acid	mg/L	<5
Lactic acid	mg/L	5200

6.2 Phase 1 – Batch Trials without Inoculum (Month 6–10)

Based on the scientific literature review (section 4), a laboratory-scale reactor configuration was designed and implemented for the valorisation of cheese whey into VFAs. Initial trials were conducted in batch mode at various HRTs (2, 5 and 10 days) to assess baseline performance. During early batch trials, nitrogen gas was supplied after raw whey’s feeding to rapidly establish anaerobic conditions, as confirmed by monitoring the oxidation-reduction potential (ORP) in both Squacquerone and Ricotta whey matrices. In later stages, however, experiments at HRT = 10 days were carried out without nitrogen purging, as the system naturally developed and maintained sufficiently low redox potentials, eliminating the need for oxygen removal by nitrogen purging. Results of phase 1 trials are presented below:

HRT: 2 days

Trials conducted with an HRT of 2 days yielded negligible concentrations of VFAs in both Squacquerone and Ricotta cheese whey. In the Squacquerone whey trial, acetic acid concentration was 140 mg/L, while the Ricotta sample showed no detectable concentrations of acetic acid or other VFAs.

Furthermore, both substrates revealed high concentrations of lactic acid, with evident levels of lactose in the ricotta trial, thus potentially suggesting a need for longer retention times to achieve complete acidogenic fermentation, as presented in Figure 2 and Figure 3.

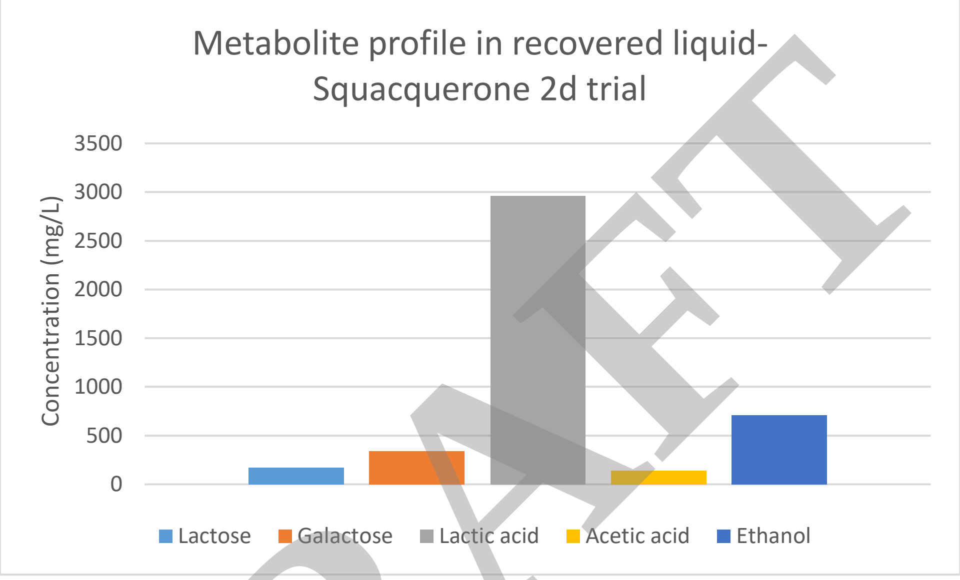

Figure 2 Metabolite profile- Squacquerone 2d trial.

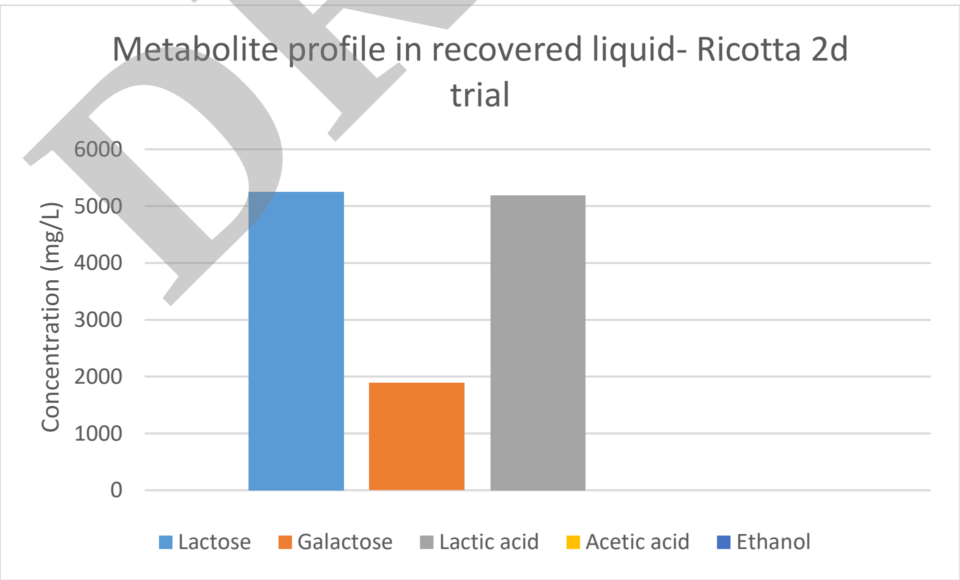

Figure 3 Metabolite profile- Ricotta 2d trial.

HRT: 5 days

Trials conducted with an HRT of 5 days likewise failed to yield an evident concentration of VFAs in either Squacquerone or Ricotta cheese whey. However, the Squacquerone sample exhibited a modest increase, with acetic acid reaching approximately 710 mg/L, while the Ricotta sample produced around 320 mg/L.

Still, both substrates remained rich in lactic acid, highlighting the incomplete fermentation. These results suggested that much longer SRTs are needed to fully break down the substrates, especially given the high levels of lactic acid still present in both whey types, to investigate their fate for conversion into VFA. The metabolite profile of both trials is presented in Figure 4 and Figure 5.

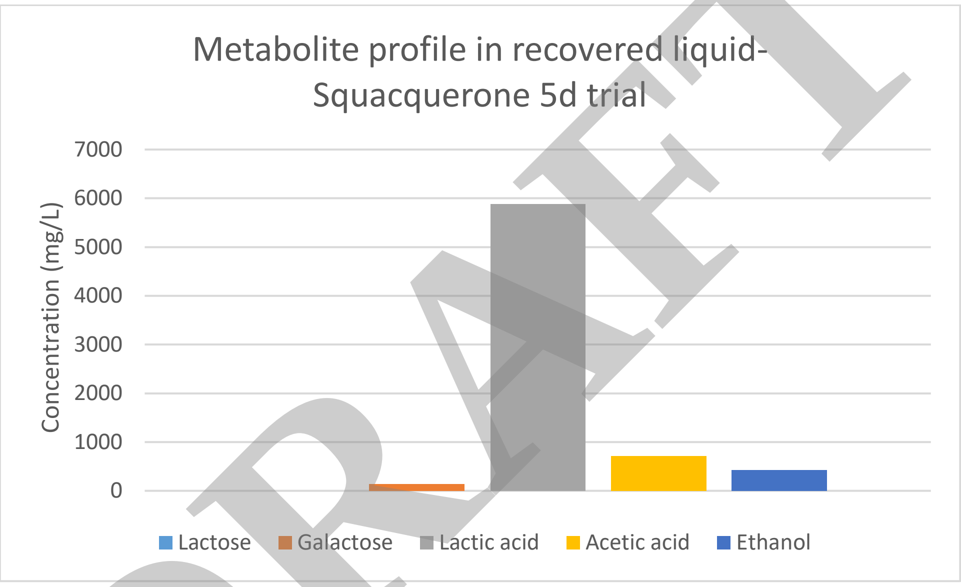

Figure 4 Metabolite profile- Squacquerone 5d trial.

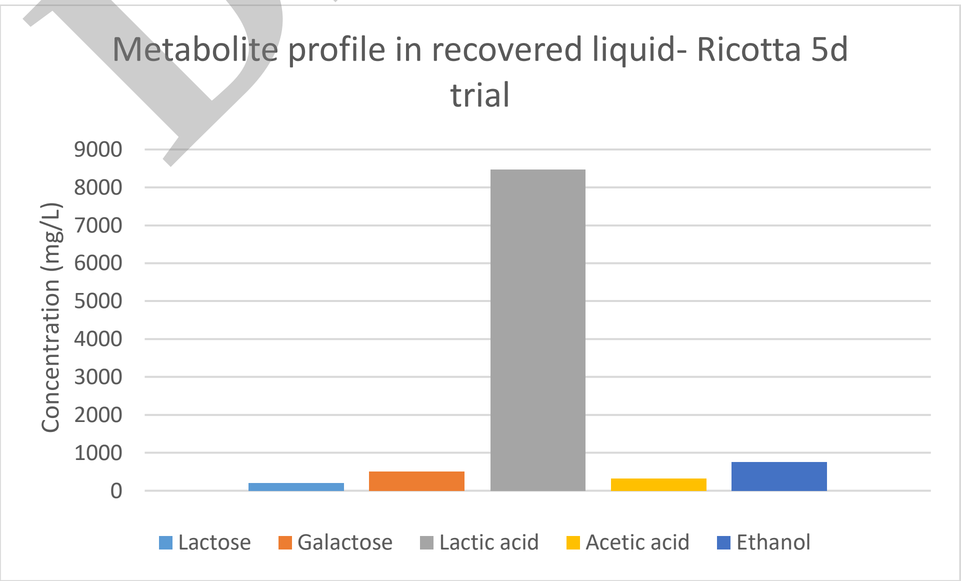

Figure 5 Metabolite profile- Ricotta 5d trial.

HRT: 10 days

A 10-day HRT trial was carried out only with Squacquerone cheese whey to examine how extended retention times affect substrate consumption and VFA production. Samples were taken at various stages during the process to monitor changes in lactose, lactic acid, and the yield and composition of VFAs (Figure 6). Recovered liquid was post-treated by centrifugation plus 0.45-micron filtration to evaluate a suitable post-treatment strategy for VFA recovery.

Throughout the trial, lactose levels gradually declined, yet roughly half of the initial amount remained unconsumed by the end. Lactic acid was found in high concentrations within the final recovered liquid, suggesting favourable conditions for lactic acid bacteria at uncontrolled pH for lactose conversion. Notably, the VFA profile was composed of approximately 1500 mg/L of acetic acid, 300 mg/L of propionic acid, and 850 mg/L of butyric acid in the final recovered liquid that was post-treated by centrifugation followed by 0.45-micron filtration, highlighting its suitability for VFA recovery, as compared to centrifugation only.

Finally, these results emphasised the need to investigate semi-batch operation mode at controlled pH (6 & 7), higher retention times and commercial bacterium strains as inoculum to understand their influence on VFA yield and process optimisation.

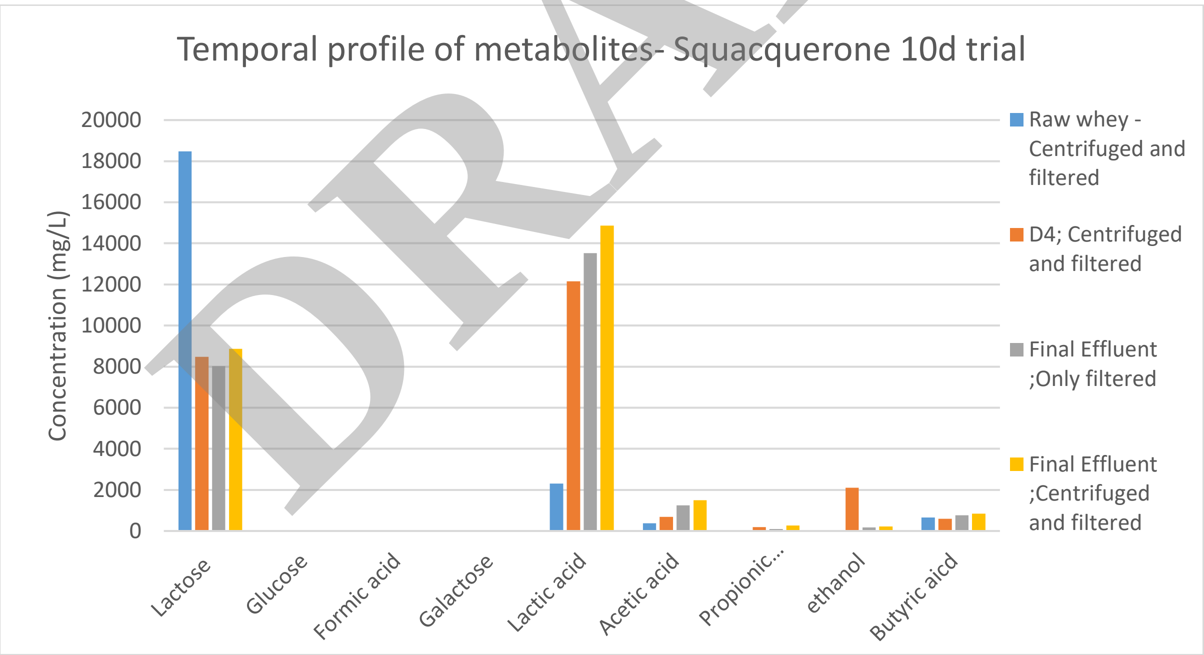

Figure 6 Temporal variation of metabolite profile- Squacquerone 10d trial

6.3 Iterative Testing and Optimisation (Month 10-15)

Between months 10 and 13, optimisation was intensified, focusing on maximising VFA concentration through modifications to the operational strategy, including strict pH control, testing of commercial inoculum alongside native consortia, and the transition to semi-batch operation (Phases 2 and 3) to enhance process control and VFA yields, while addressing operational constraints.

6.3.1 Phase 2

The results from Phase 2, operated in semi-batch mode at higher solid and hydraulic retention times (SRT 35 days & HRT 10 days), were run to assess whether prolonged SRTs would support biomass acclimation and enrichment to improve substrate conversion at a manually controlled pH of 6. Two trials, one with the addition of inoculum (*Propionibacterium*) and another as a control (without inoculum), were conducted, with process conditions, results and discussion given below.

Table 12. Process conditions of phase 2.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	Days	35
HRT	Days	10
pH	-	6

***Propionibacterium strains* trial:** The trial using the *Propionibacterium* demonstrated an evident increase in both VFA yield and lactic acid breakdown. After 14 days of operation, VFA levels reached a peak of around 17 g/L. From there, the concentration was steady, with yield variations around 20%, indicating that the system had reached a relatively stable state and was considered operating under steady-state conditions.

The recovered VFA liquid is mostly composed of butyric acid, making up more than 66% of the total, followed by propionic acid at 25% and a smaller proportion of acetic acid at 8%. Importantly, lactic acid levels dropped sharply and stayed below 300 mg/L after day 12, indicating that VFA-producing bacteria used it as a carbon source for volatile fatty acids production.

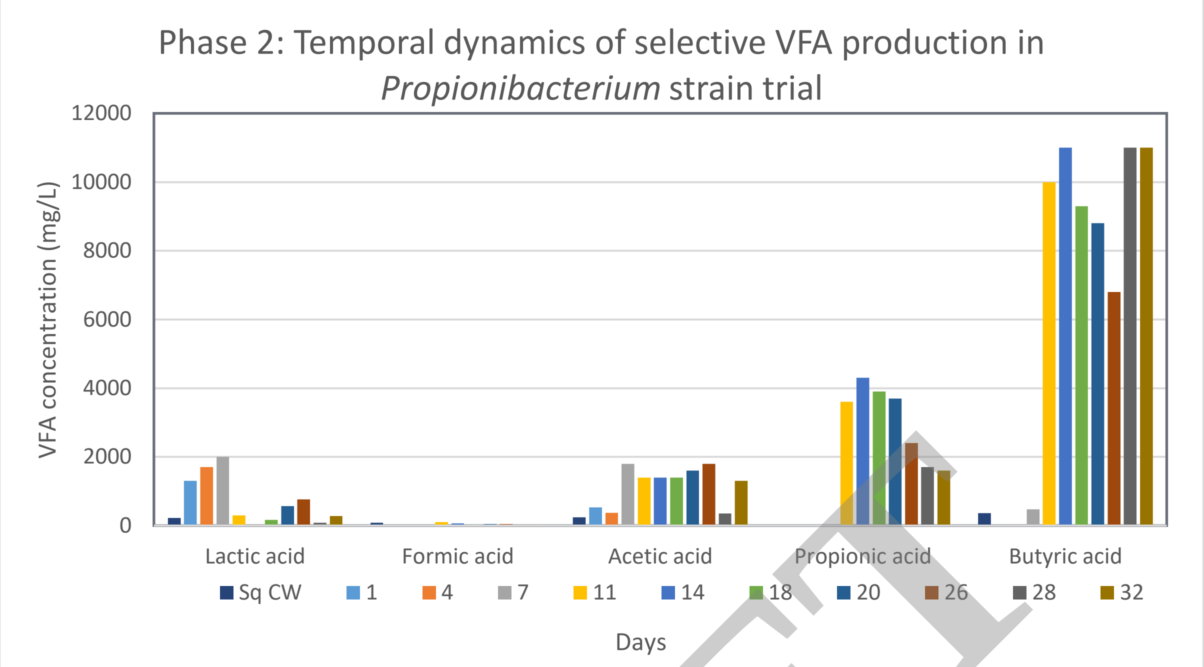

Figure 7. Selective VFA production over time during Phase 2- Propionibacterium trial.

The selective VFA profile obtained during the *Propionibacterium*-inoculated semi-batch trial is presented in Figure 7. The initial composition of the Squacquerone cheese whey (day 0, dark blue bars) showed a lactic acid concentration of 220 mg/L, alongside low background levels of VFAs, particularly butyric (360 mg/L), acetic (240 mg/L) and formic acids (85 mg/L), while propionic acids was essentially absent.

Over the course of the trial, a clear metabolic shift was observed. Lactic acid was rapidly formed until day 7, reaching a concentration of 2000 mg/L, and then consumed, with concentrations dropping below 300 mg/L after day 11 and remaining low until the end of the experiment, indicating that lactic acid served as a primary carbon source for microbial acidogenesis. Simultaneously, a progressive accumulation of VFAs occurred.

Among the acids produced, butyric acid showed the most significant increase, rising from undetectable levels at day 0 to concentrations exceeding 11000 mg/L by day 32, confirming its role as the main fermentation product under the given operational conditions (pH 6, mesophilic temperature, long SRT). Propionic acid also increased substantially, reaching a plateau around 3000–4000 mg/L from day 11 to 20, while acetic acid remained in the 1500–2000 mg/L range. Formic acid production was negligible throughout, with values consistently below 100 mg/L.

These results suggest that the inoculated microbial community effectively shifted the carbon flow from lactic acid towards the production of medium-chain VFAs, primarily butyrate and propionate, after a short adaptation phase. This outcome supports the hypothesis that prolonged retention times and controlled pH conditions facilitate microbial enrichment and conversion efficiency.

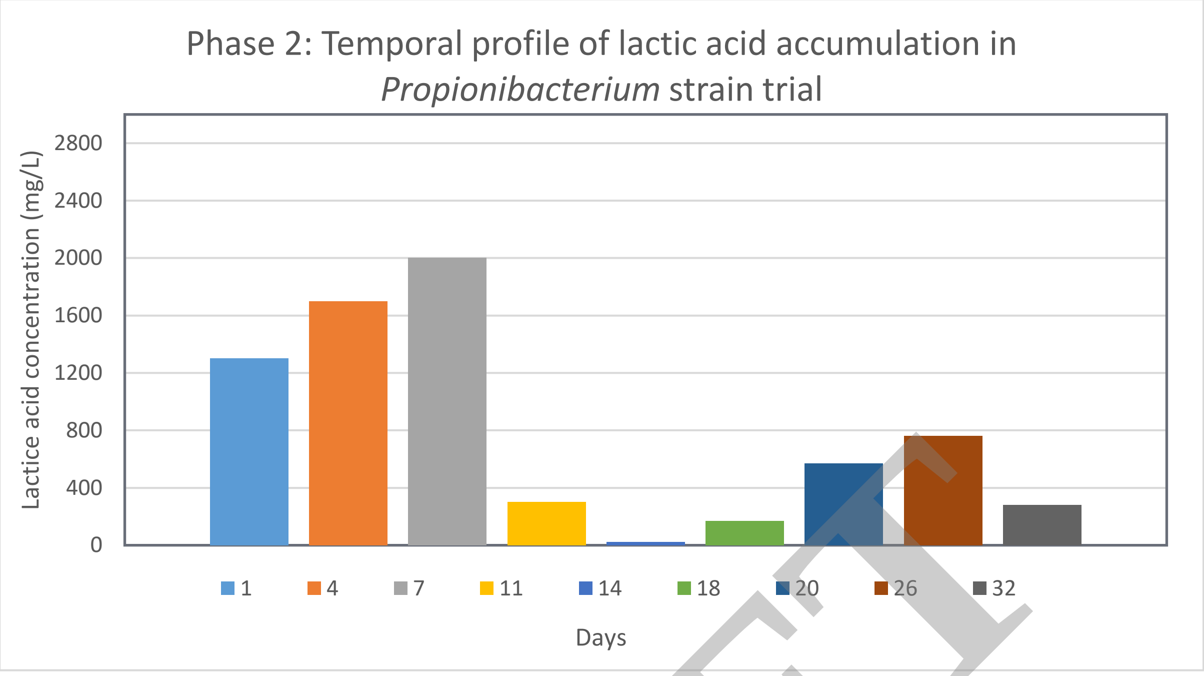

Figure 8. Lactic acid consumption over time during Phase 2- Propionibacterium trial.

The evolution of lactic acid concentration during Phase 2 in the Propionibacterium-inoculated trial is depicted in Figure 8. Initially, lactic acid levels increased, reaching a peak of approximately 2000 mg/L on day 7. This transient accumulation suggests a lag phase in the activity of lactic acid-consuming bacteria, during which fermentable sugars and lactose in the Squacquerone whey may have been rapidly converted into lactic acid by co-existing lactic acid bacteria.

From day 11 onwards, a sharp decline in lactic acid concentration was observed, dropping to less than 30 mg/L by day 14 and remaining consistently low throughout the remainder of the experiment. This trend indicates that the *Propionibacterium*-based microbial community had successfully acclimated and began efficiently metabolising lactic acid into VFAs, particularly butyric and propionic acids, as shown in Figure 7.

The temporary build-up followed by rapid consumption of lactic acid reflects the dynamic metabolic balance between lactic acid producers and consumers within the reactor. The effectiveness of this transition suggests that the chosen operational conditions—especially the low pH and extended SRT—were favourable for the development of an acidogenic microbial community capable of driving secondary fermentation processes.

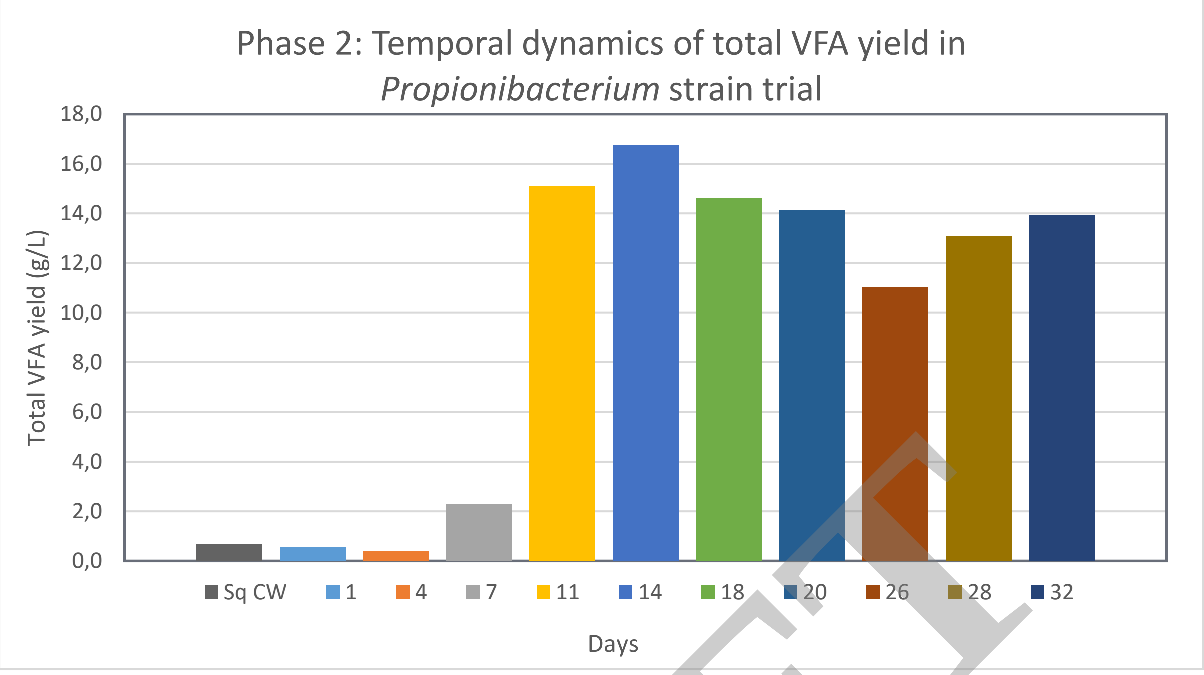

Figure 9. Total VFA yield over time during phase 2- Propionibacterium trial.

The total VFA yield obtained over time in the Propionibacterium-inoculated trial is reported in Figure 9. The raw Squacquerone cheese whey exhibited negligible initial VFA content (<1 g/L), confirming that the fermentation products observed are the result of active microbial conversion processes.

A substantial increase in VFA yield was observed between day 7 and day 11, reaching approximately 15 g/L by day 11. This rapid rise aligns with the observed drop in lactic acid (Figure 8), indicating the onset of secondary fermentation processes driven by acidogenic bacteria. The yield peaked at 16.8 g/L on day 14, suggesting optimal conversion efficiency during this period.

Following this peak, VFA concentrations remained relatively stable, fluctuating within a narrow range (11–15 g/L) until day 32. This behaviour indicates that the system had entered a quasi-steady-state, where daily substrate input and microbial activity were balanced, sustaining high VFA levels with minimal variation.

These results confirm that under the selected semi-batch operating conditions (pH 6, SRT 35 days), the microbial community enriched with *Propionibacterium* was capable of achieving and maintaining high VFA yields, with butyric and propionic acids as the main contributors, as also shown in Figure 7. The sharp increase followed by yield stabilisation also reflects the successful adaptation of the biomass and the effective conversion of both residual lactose and lactic acid into target fermentation products.

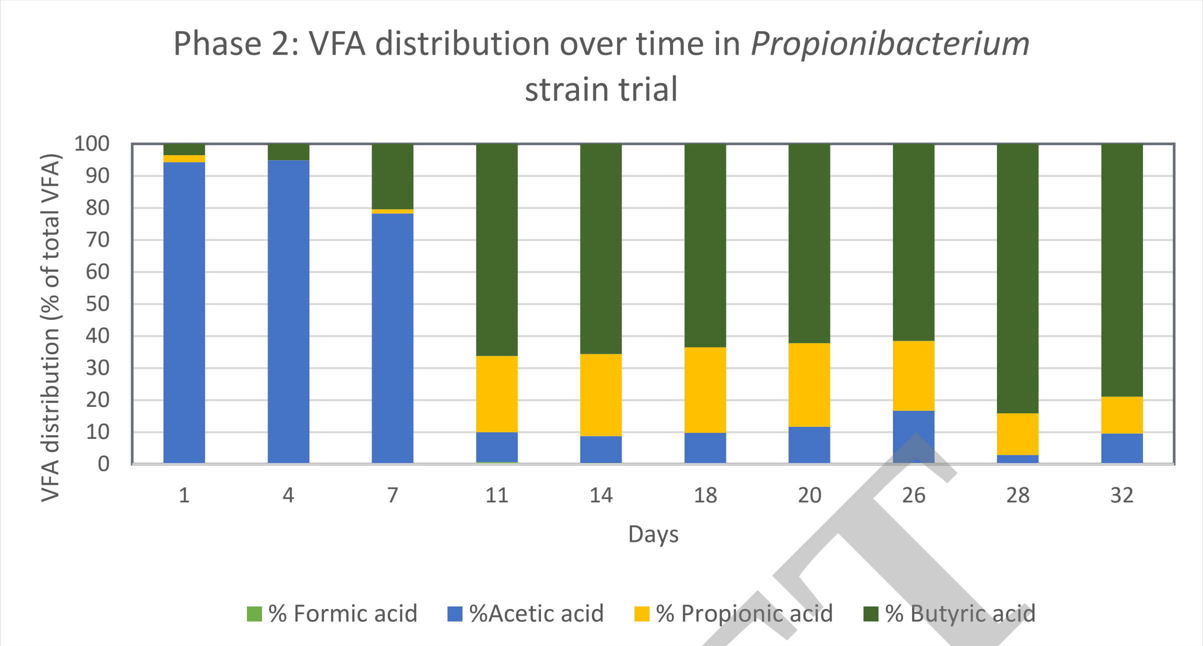

Figure 10. VFA distribution over time during phase 2- Propionibacterium trial.

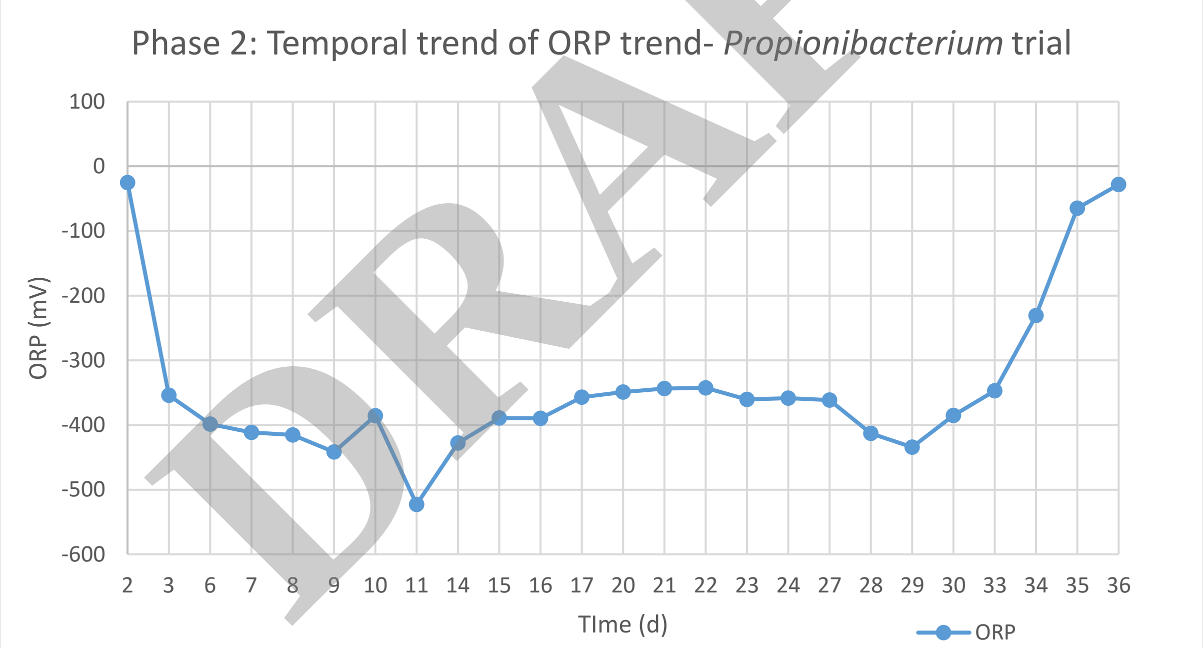

Figure 11. Temporal variation of ORP during phase 2- Propionibacterium trial.

The evolution of VFA distribution during the *Propionibacterium* strain trial is illustrated in Figure 10. In the early stages (days 1–7), the VFA profile was dominated by acetic acid, which accounted for over 80% of the total VFA content, while butyric and propionic acids were nearly absent. This suggests that acetic acid was likely formed during initial sugar fermentation and was already present in low amounts in the raw substrate (360 mg/L).

From day 11 onwards, a marked shift in the VFA distribution was observed. The contribution of acetic acid dropped significantly, stabilising around 10–15%, while butyric acid emerged as the dominant product, consistently representing over 60% of the total VFA content. In parallel, propionic acid increased, reaching 25–30% of the total VFA fraction. Formic acid, although always present in minor proportions, remained relatively constant below 5%.

This change in distribution confirms a functional transition in the microbial community, likely driven by the selective enrichment of butyrate- and propionate-producing bacteria, including the inoculated Propionibacterium. The stable dominance of butyric acid over the second half of the experiment reflects favourable metabolic conditions (low pH, anaerobiosis, long SRT) that promote solventogenic pathways over acidogenic ones.

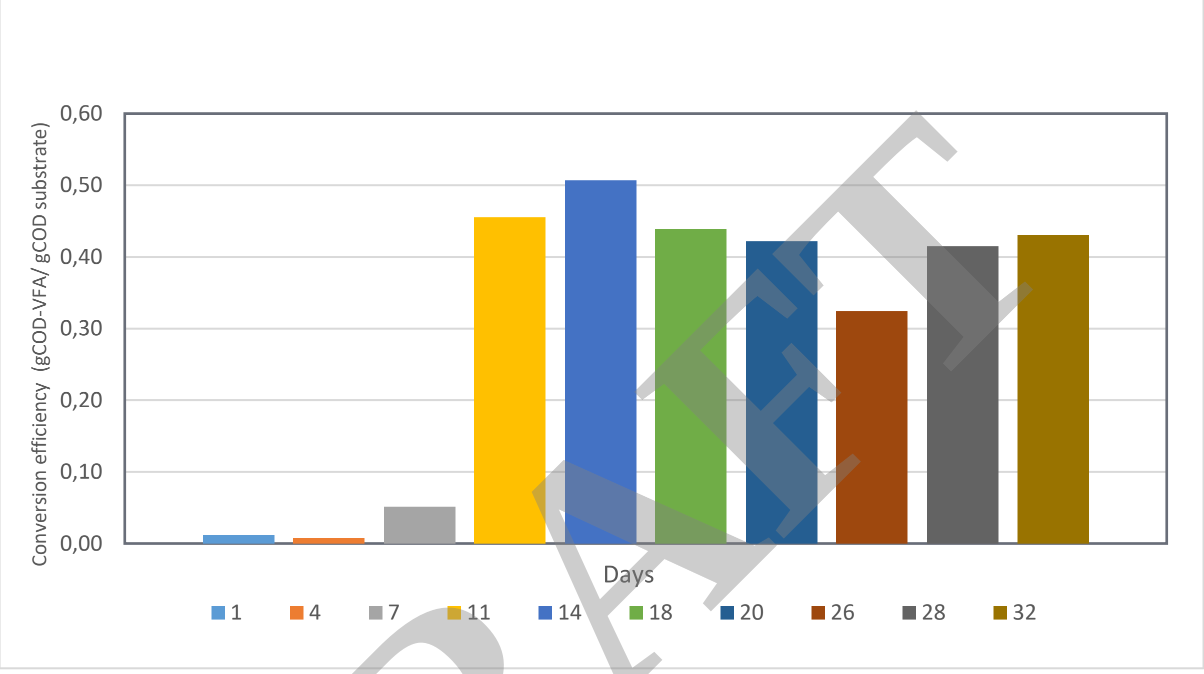

Figure 12. Process substrate conversion efficiency over time during phase 2- Propionibacterium trial.

The process conversion efficiency during the *Propionibacterium*-inoculated trial, expressed as gCOD of VFAs produced per gCOD of substrate fed, is shown in Figure 12. A very low conversion efficiency was observed in the initial stage (days 1–7), consistent with the lag phase of microbial adaptation and the initial accumulation of lactic acid as an intermediate metabolite (Figure 8).

Starting from day 11, a substantial increase in conversion efficiency was observed, peaking at 0.51 gCOD-VFA/gCOD-substrate on day 14, which coincides with the highest total VFA concentration recorded (Figure 9). This confirms that the system had reached its maximum substrate conversion capacity during this period, indicating optimal microbial activity under the established operating conditions.

Following the peak, the efficiency declined slightly and then stabilised around 0.42–0.44 gCOD-VFA/gCOD-substrate from day 18 onwards. The temporary dip to 0.33 gCOD-VFA/gCOD-substrate on day 26 may be attributed to minor fluctuations in biomass activity or substrate variability, but the system recovered rapidly, maintaining a relatively stable performance over time.

Overall, these results demonstrate that the *Propionibacterium*-enriched microbial community was able to convert a substantial fraction of the available substrate into VFAs, achieving a conversion efficiency close to 50%. The process thus shows a potential for dairy whey conversion into VFA by acidogenic fermentation. However, further optimisation strategies may be investigated to underpin process efficiency for enhanced VFA production.

Temporal trend of oxidation-reduction potential (ORP) during Phase 2 (*Propionibacterium* trial). Throughout the trial, ORP values remained consistently negative, generally between –400 and –500 mV indicating the maintenance of stable anaerobic conditions required for acidogenic fermentation. The slight fluctuations observed, particularly the peak negative excursion on day 12 and the rise after day 30, likely reflect substrate loading dynamics and microbial activity shifts. Nonetheless, the overall profile confirms that the process operated within a strongly reducing environment conducive to volatile fatty acid production.

Control trial: Similarly, the control trial, conducted without the addition of the inoculum, also showed noticeable improvement in both VFA yield and lactic acid consumption. After 21 days of operation, VFA concentrations peaked at around 14 g/L, then gradually settled into a range between 11 and 13.5 g/L.

The VFA profile was like the inoculated trial, dominated by butyric acid, which made up over 87% of the total. Propionic acid was present at a lower level (4.5%), with acetic acid contributing around 8%. Lactic acid levels steadily declined and remained below 500 mg/L after 15 days, suggesting that even without external microbial addition, the native microbial consortium was capable of VFA production, although at a slower pace and yield than the inoculated trial.

The trial with the Propionibacterium achieved a higher VFA concentration yield in a shorter time, reaching 17 g/L in 15 days compared to 14 g/L in 21 days in the control. While the inoculated system produced a slightly high VFA concentration, the control trial also showcases the potential of the native microbial community for VFA yield.

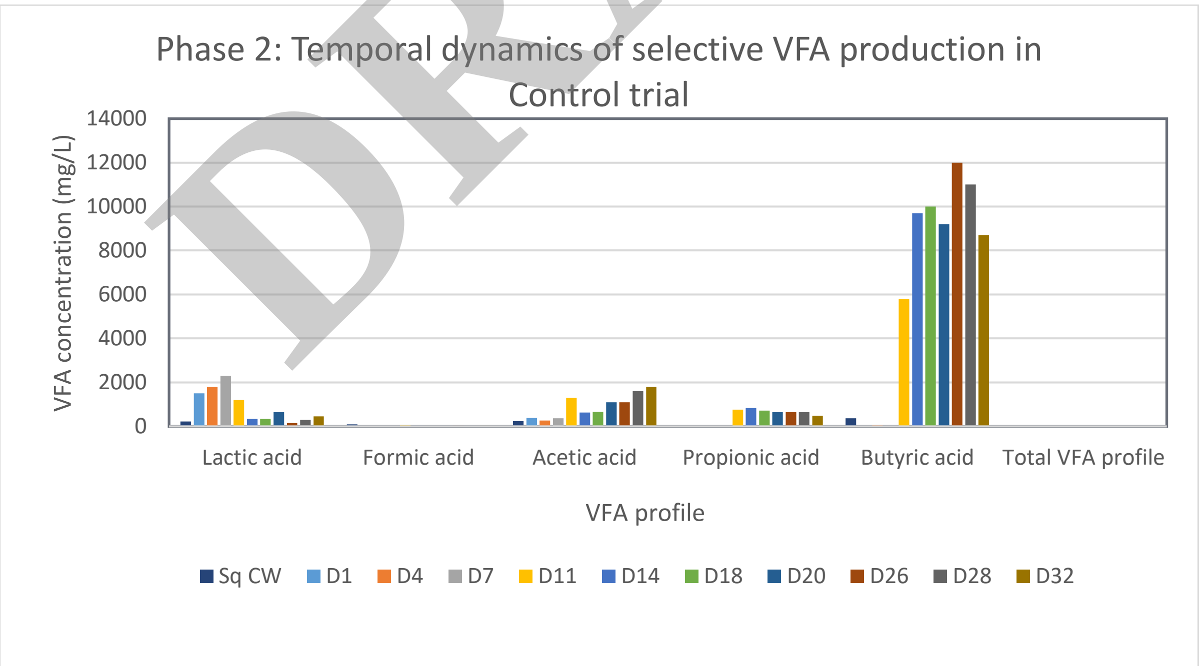

Figure 13. Selective VFA production over time during Phase 2- Control trial.

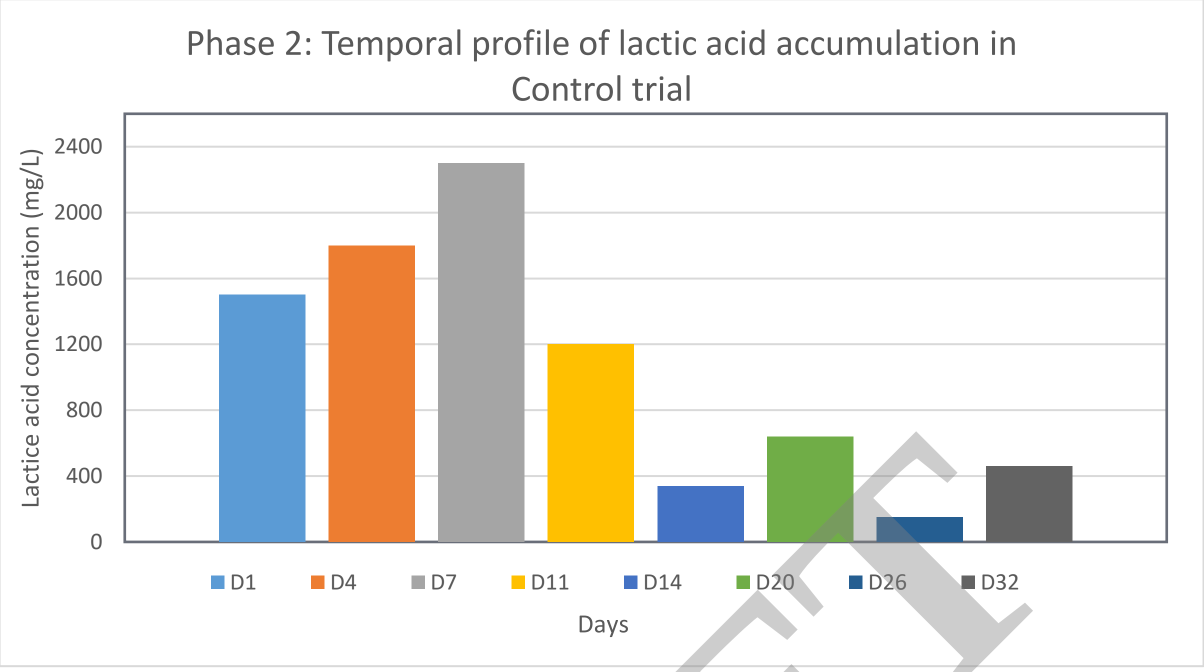

Figure 14. Lactic acid consumption over time during Phase 2- Control trial

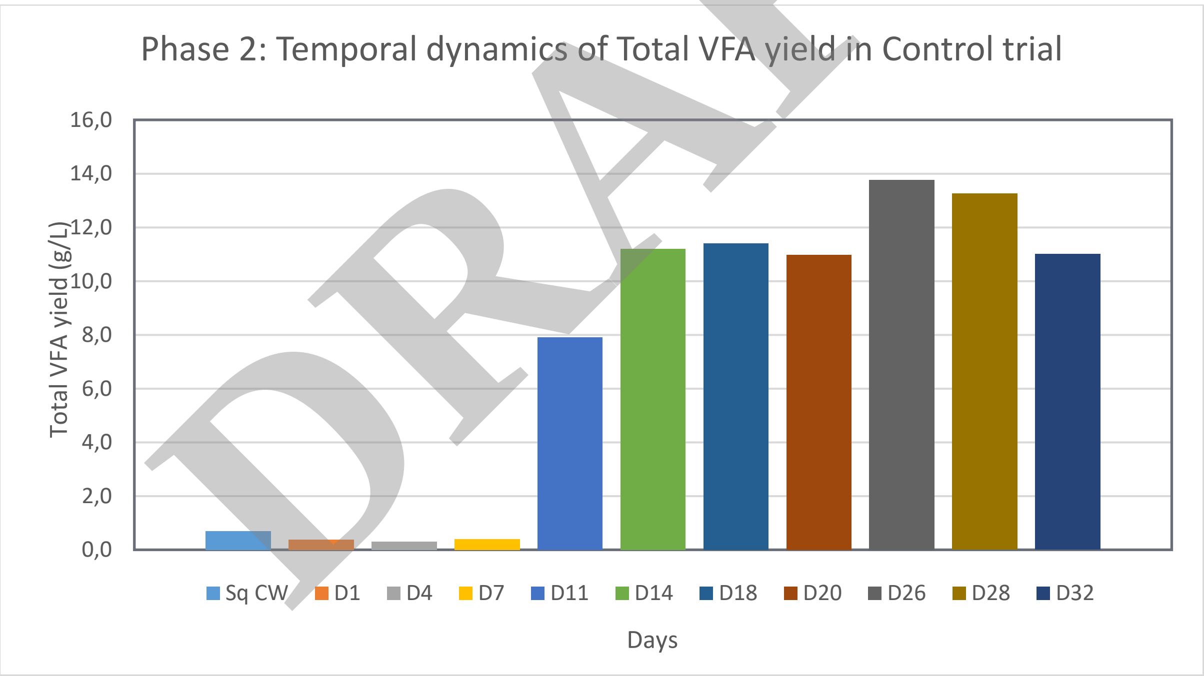

Figure 15. Total VFA yield over time during phase 2- Control trial.

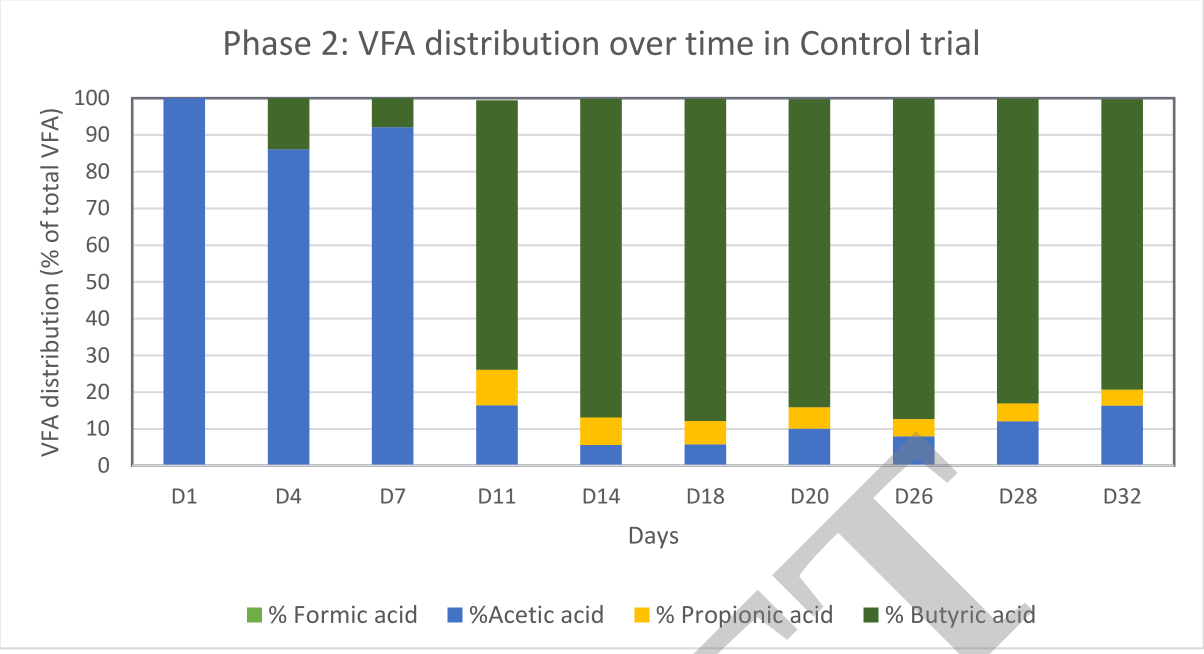

Figure 16. VFA distribution over time during phase 2- Control trial.

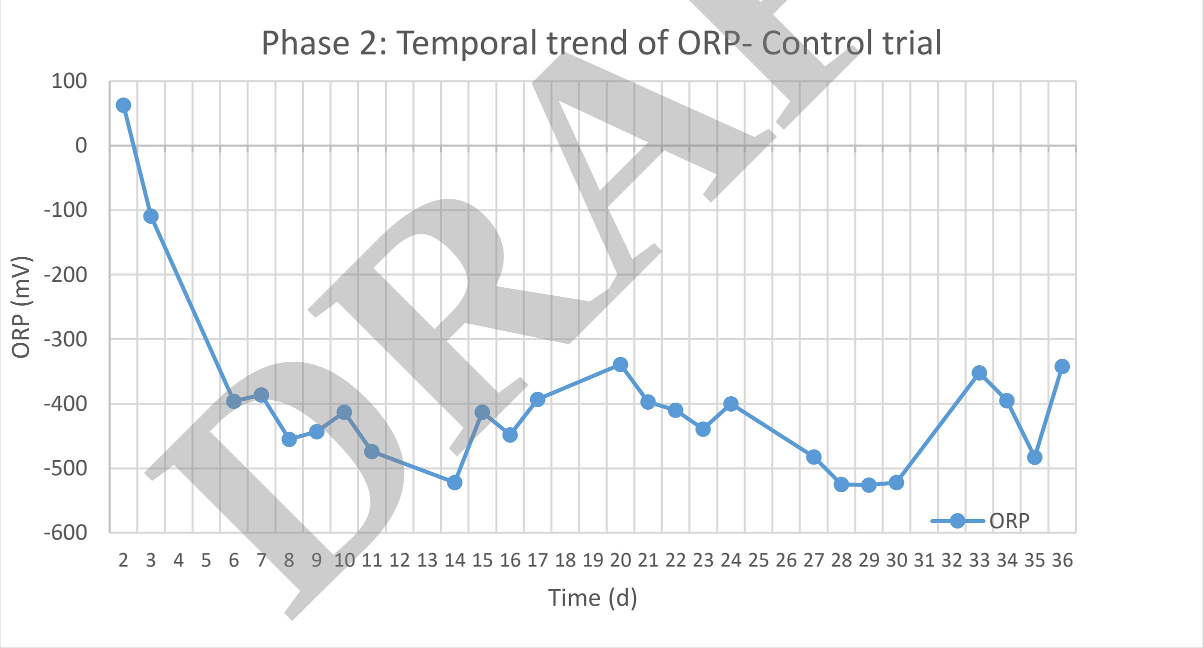

Figure 17 Temporal variation of ORP during phase 2- Control trial.

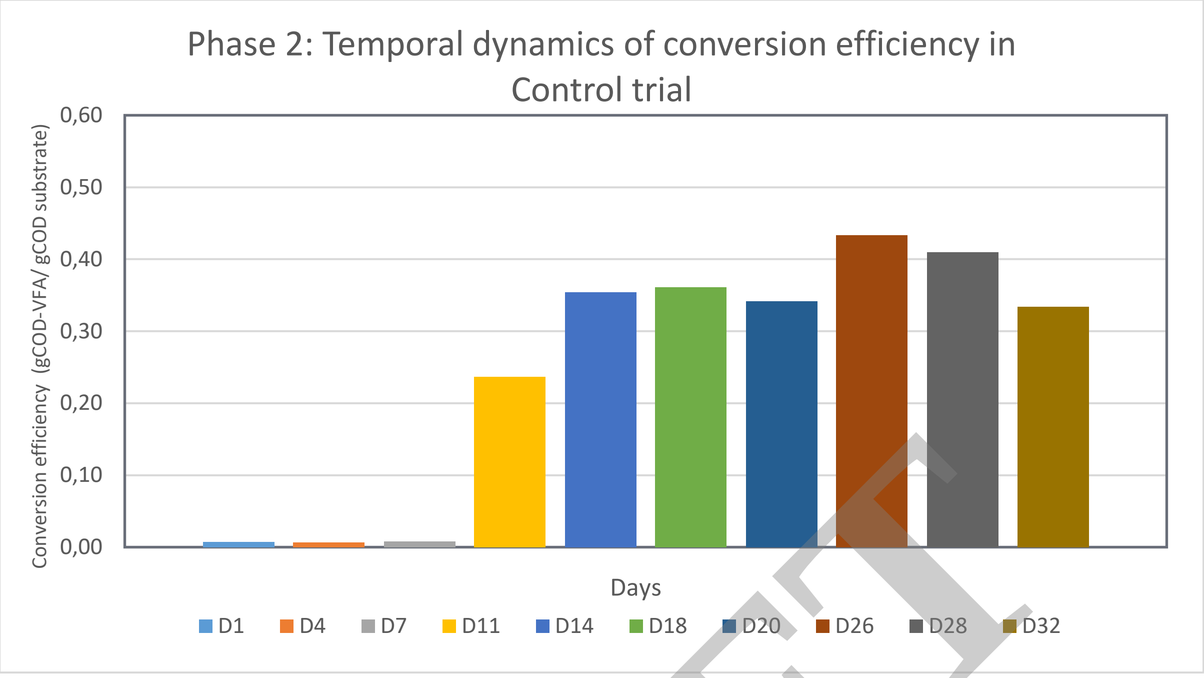

Figure 18. Process substrate conversion efficiency over time during phase 2- Control trial.

The control trial, conducted under the same operating conditions as the inoculated setup (semi-batch mode, 37 °C, SRT = 35 days, HRT = 10 days, pH 6), aimed to evaluate the fermentative potential of the native microbiota naturally present in Squacquerone cheese whey (Figure 13). Despite the absence of a dedicated inoculum, the system demonstrated a clear capacity for substrate conversion and VFA production, albeit with different kinetics and distribution profiles compared to the *Propionibacterium*-enriched reactor.

As shown in Figure 14, the concentration of lactic acid initially increased, reaching a peak of 2300 mg/L by day 7, due to the rapid activity of indigenous lactic acid bacteria. From day 11 onward, lactic acid concentrations declined steadily, dropping below 400 mg/L by day 14 and remaining low, indicating that endogenous acidogenic microbes progressively shifted towards the consumption of lactate and its conversion into VFAs.

The corrected VFA production profile (Figure 13) reflects this dynamic: butyric acid emerged as the dominant product, reaching a maximum concentration of 12000 mg/L on day 26, while propionic and acetic acids remained at much lower levels, generally below 1000 mg/L. Formic acid was produced in negligible amounts.

These findings are confirmed by the overall VFA yield trend (Figure 15), which showed a progressive increase from day 11 onwards, stabilising between 11 and 14 g/L between days 20 and 28. This plateau phase suggests that a quasi-steady-state condition was reached, where substrate input and microbial conversion were balanced.

The VFA distribution (Figure 16) showed limited variability over time. From day 11 onward, butyric acid consistently accounted for over 70% of the total VFAs, while propionic and acetic acids remained minor fractions, typically below 15% each. Compared to the inoculated trial, this indicates a less diversified product spectrum, with a clear dominance of butyrate-producing pathways and limited propionate generation, likely due to the absence of *Propionibacterium*.

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Finally, conversion efficiency in terms of COD balance (Figure 18) reached a maximum of approximately 0.43 gCOD-VFA/gCOD-substrate around day 26, slightly below the 0.51 observed in the inoculated trial. After peaking, the efficiency remained stable, reflecting consistent performance of the microbial community.

Figure 17 shows that the ERP in the control trial also remained within a consistently negative range (typically between –400 and –500 mV), indicating that anaerobic conditions were successfully maintained throughout the experiment even in the absence of inoculum. This stability in ORP suggests that the endogenous microbial community was able to preserve the reductive environment necessary for acidogenic activity.

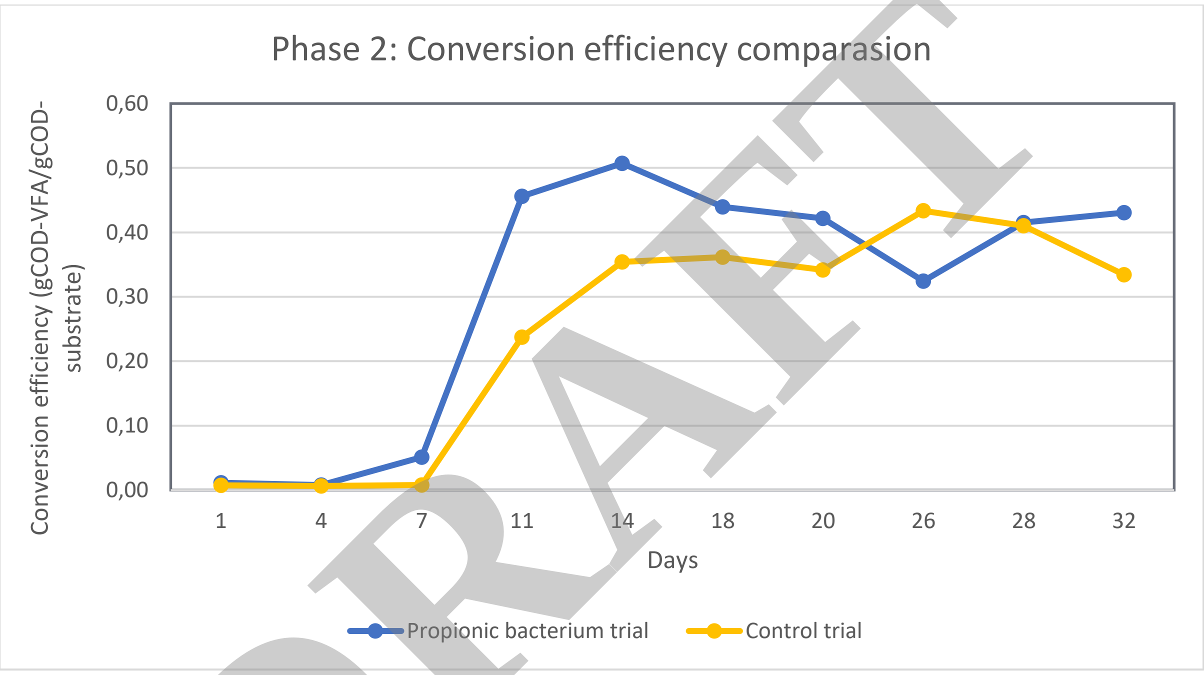

Figure 19. Comparison of substrate conversion efficiency during phase 2- Propionibacterium vs. Control trial.

Figure 19 provides a direct comparison of the substrate conversion efficiency between the inoculated (*Propionibacterium*) and control (no inoculum) trials over the course of Phase 2. The trends reveal notable differences in process kinetics, peak performance, and stability over time.

The *Propionibacterium*-inoculated system showed a rapid increase in efficiency, jumping from near-zero to approximately 0.45 gCOD/gCOD between days 7 and 11, and reaching a maximum of 0.51 on day 14. This sharp rise indicates faster acclimation and enrichment of the microbial community when supported by the selected inoculum, leading to earlier and more effective conversion of lactic acid and residual sugars into VFAs. After the peak, efficiency declined slightly but remained consistently above 0.40, indicating a stable and robust fermentation process.

In contrast, the control trial exhibited a slower and more gradual increase, reaching its peak efficiency of 0.43 gCOD/gCOD on day 26, a delay of nearly two weeks compared to the inoculated setup. This suggests that, although the native microbiota can eventually adapt and achieve comparable conversion levels, the lag phase is more pronounced, and the overall microbial activity is slightly less efficient in the absence of targeted inoculation. Additionally, the control trial displayed greater variability in the final stages, with efficiency dropping to around 0.33 by day 32.

Overall, the comparison confirms that the addition of *Propionibacterium* significantly accelerates process start-up, improves early-stage substrate utilisation, and supports higher peak and sustained efficiencies. While both systems achieved meaningful conversion of organic matter to VFAs, inoculation provided advantages in terms of kinetics and process stability, which are especially relevant for scaled-up applications where reactor throughput and operational timeframes are critical.

6.3.2 Phase 3

Phase 2 demonstrated that extended solids retention times and controlled pH improved process efficiency. However, it was also considered that extended retention could potentially lead to biomass ageing or reduced microbial activity, resulting in lower concentrations of active biomass.

In Phase 3, the process was further optimised by reducing the SRT to 20 days while maintaining a neutral pH (7) and constant hydraulic retention time (10 days). The objective was to examine whether a shorter SRT would sustain or enhance active biomass levels by minimising biomass decay or washout. The process conditions, as well as results and discussion of these trials, are presented below.

Table 13. Process conditions of phase 3.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	20
HRT	days	10
pH	-	7

Propionibacterium strains trial

Propionibacterium trial at pH 7 showed further increase in VFA accumulation and lactic acid breakdown. Maximum VFA yield (>23 g/L) was observed at around 4 weeks of operation, with A comparable VFA yield at 14 days, inferring that shorter operation cycles are substantial for VFA accumulation and production. Besides, from day 14 until day 25, VFA concentration variation was lower than 3% indicating that steady state conditions are obtained.

The recovered VFA liquid was mainly composed of butyric acid, making up more than 84% of the total, followed by acetic acid at 11.5% and a smaller proportion of propionic acid at 4.5%. Importantly, lactic acid levels dropped sharply and stayed below 250 mg/L after day 11, a potential indication that VFA-producing bacteria were actively using it as a carbon source to convert it into valuable VFAs.

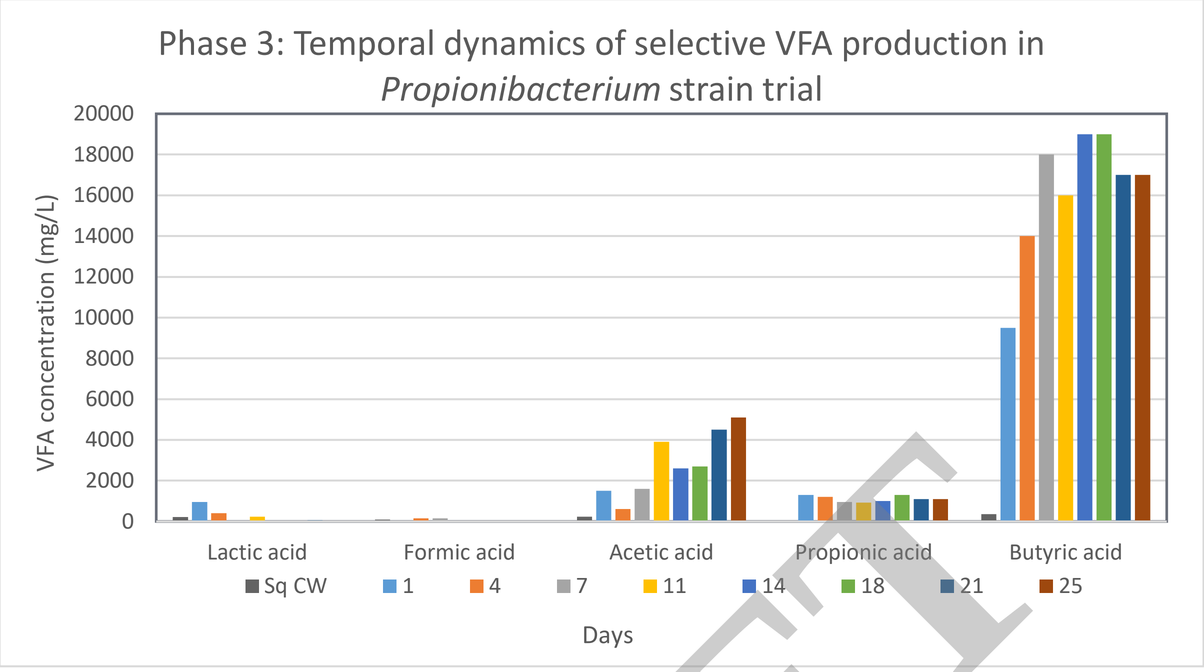

Figure 20. Selective VFA production over time during Phase 3- Propionibacterium trial.

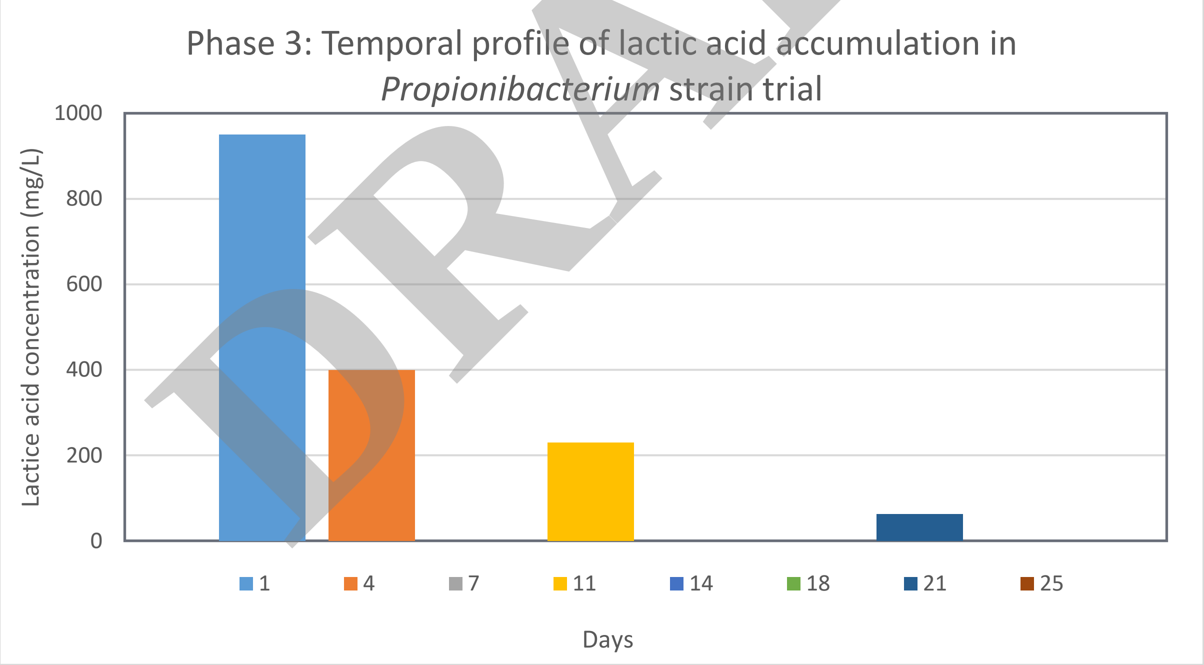

Figure 21. Lactic acid consumption over time during Phase 3- Propionibacterium trial.

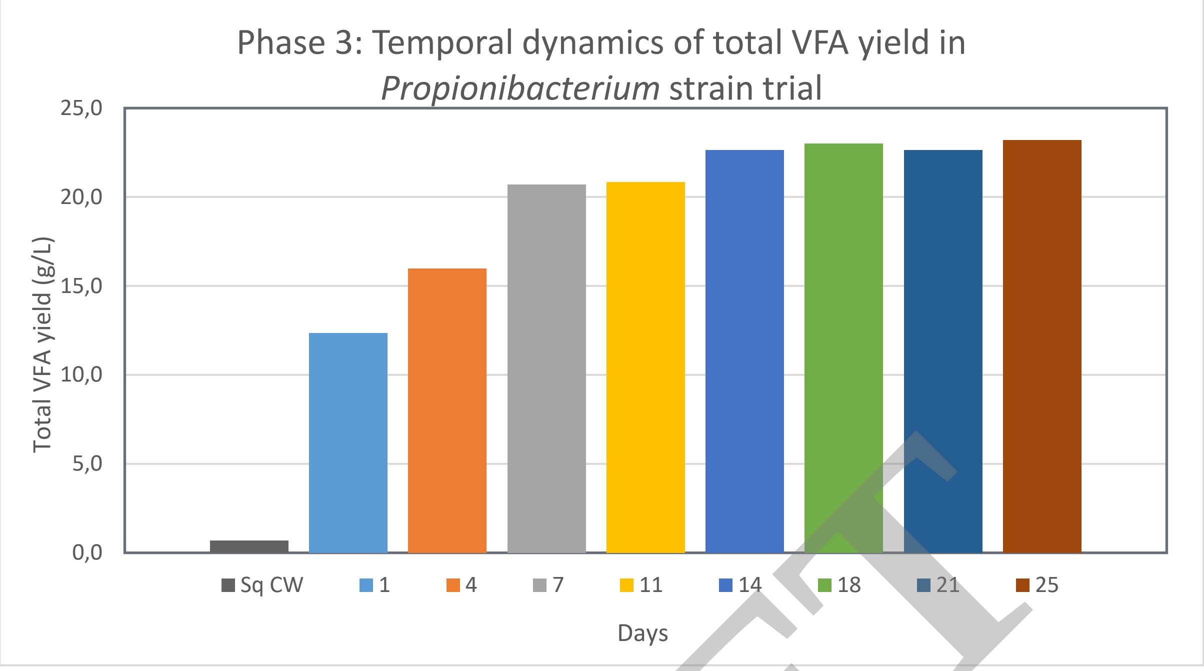

Figure 22. Total VFA yield over time during phase 3- Propionibacterium trial.

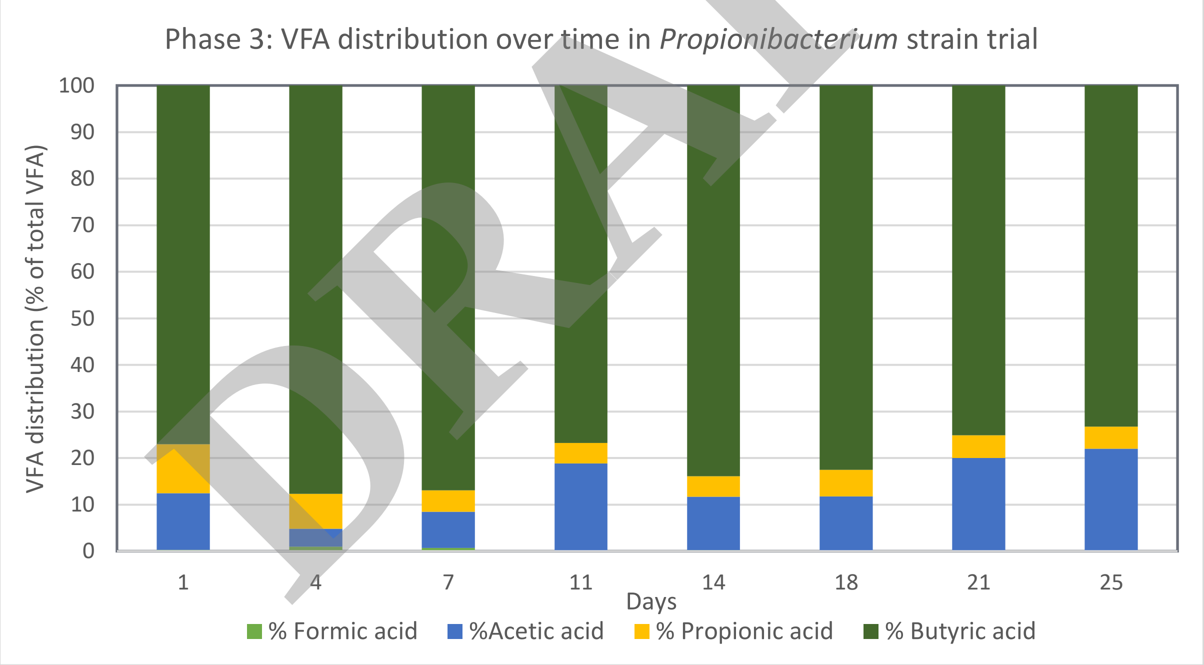

Figure 23. VFA distribution over time during phase 3- Propionibacterium trial.

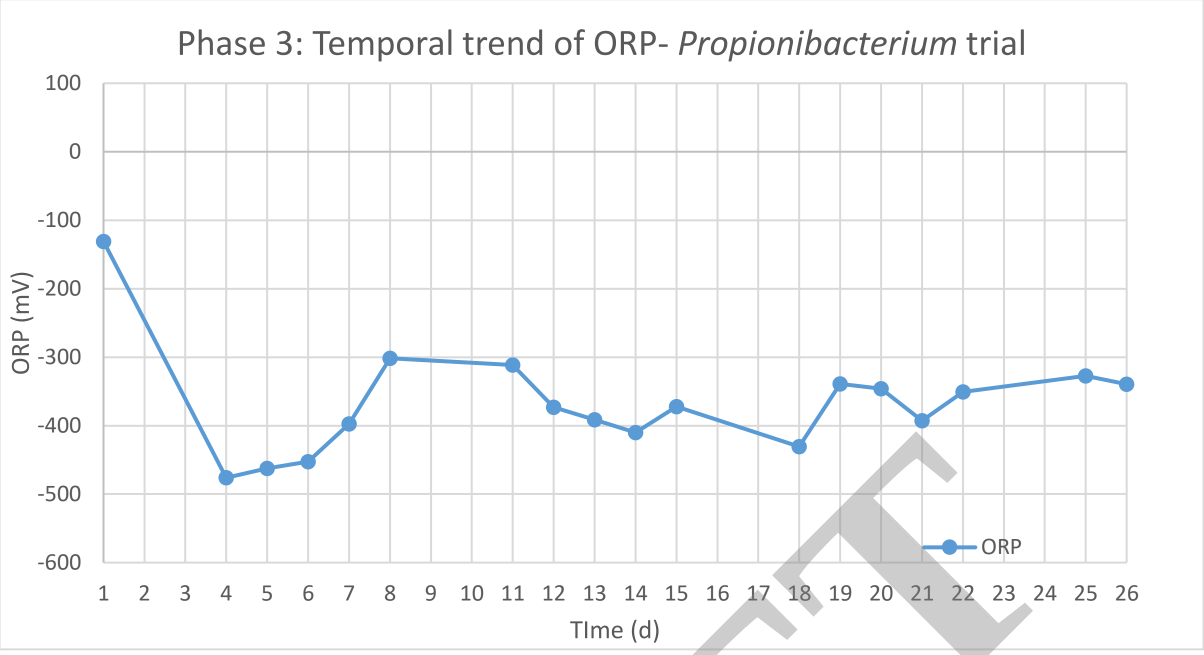

Figure 24 Temporal variation of ORP during phase 3- Propionibacterium trial.

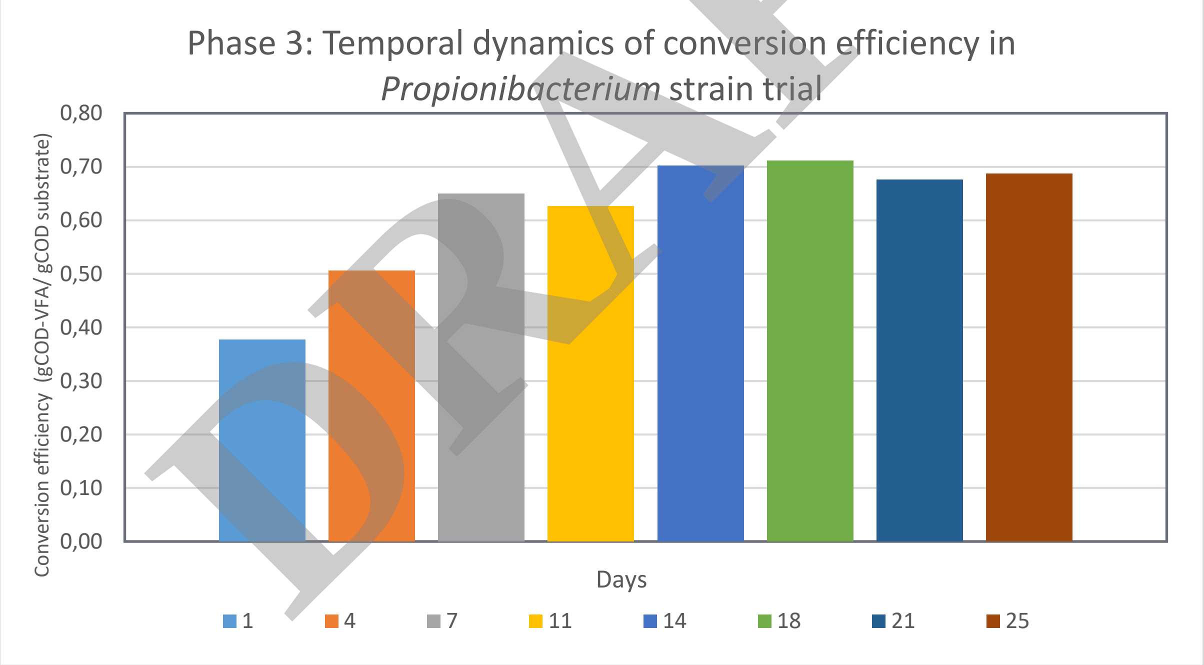

Figure 25. Process substrate conversion efficiency over time during phase 3- Propionibacterium trial.

The results, summarised in Figures 13–17, reveal a marked improvement in both process performance and substrate conversion efficiency.

As shown in Figure 20, butyric acid rapidly became the dominant fermentation product, reaching concentrations close to 19000 mg/L by day 15—significantly higher than the peaks observed in Phase 2. Acetic acid was also produced in substantial amounts, reaching over 5000 mg/L by the end of the trial, while propionic acid remained limited, with concentrations consistently below 1500 mg/L. This shift suggests that neutral pH conditions may have suppressed propionic acid synthesis pathways

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typically associated with *Propionibacterium*, favouring instead butyrogenic and acetogenic metabolism.

Lactic acid dynamics, shown in Figure 21, support this interpretation: although the initial concentration was already modest (950 mg/L on day 1), lactic acid was efficiently consumed, dropping below 250 mg/L by day 11 and remaining at trace levels for the remainder of the experiment, indicating a well-adapted microbial community capable of rapidly converting lactate into VFAs.

The total VFA yield, presented in Figure 22, increased steadily until day 7 and then stabilised between 22 and 24 g/L, maintaining this level through day 25. This suggests that the system achieved a stable operating regime early in the process, likely facilitated by enhanced microbial activity and efficient substrate utilisation under the neutral pH and shorter SRT.

The VFA distribution profile (Figure 23) remained stable over time, with butyric acid accounting for 77–88% of total VFAs, acetic acid contributing 4–18%, and propionic acid representing only a minor share (4–10%). This confirms that pH 7 strongly favoured butyrate-producing metabolic pathways, even in the presence of *Propionibacterium* inoculum. Finally, process efficiency, shown in Figure 25, improved significantly compared to previous trials: conversion efficiency reached 0.51 gCOD/gCOD by day 4, peaked at 0.71 by day 18, and remained consistently high throughout the rest of the experiment. These values are among the highest observed across all phases, indicating that the combination of neutral pH and reduced biomass residence time not only accelerated microbial acclimation but also enhanced the conversion of organic substrate into valuable fermentation products.

Figure 24 illustrates the temporal evolution of the ORP during Phase 4 in the inoculated system. After an initial sharp decrease to values below –450 mV by day 4, the ORP gradually stabilised between –400 mV and –300 mV for the remainder of the experiment. This sustained negative range confirms the successful maintenance of anaerobic conditions throughout the trial, which is critical for supporting acidogenic microbial activity.

Control trial

Similarly, the control trial, conducted without the addition of the inoculum, demonstrated a noticeable improvement in both VFA production and lactic acid consumption. A maximum VFA yield (21 g/L), then gradually settled at 20 g/L at longer operations. These observations indicate that the system has reached steady-state conditions.

The VFA profile showed a similar trend, dominated by butyric acid, which made up over 87% of the total. Propionic acid was present at a much lower level (4%), with acetic acid contributing around 9%. Lactic acid levels steadily declined and remained below 10 mg/L after 11 days, suggesting that even without external inoculum, the inherent microbial consortium was capable of VFA production, with slightly lower yield than the inoculated medium.

Both trials with and without commercial inoculum demonstrated higher VFA accumulation and production at pH 7 and shorter durations. However, the inoculated trial showed a slightly higher yield than the control trial, emphasising the VFA production potential of the native microbial consortium present in cheese whey.

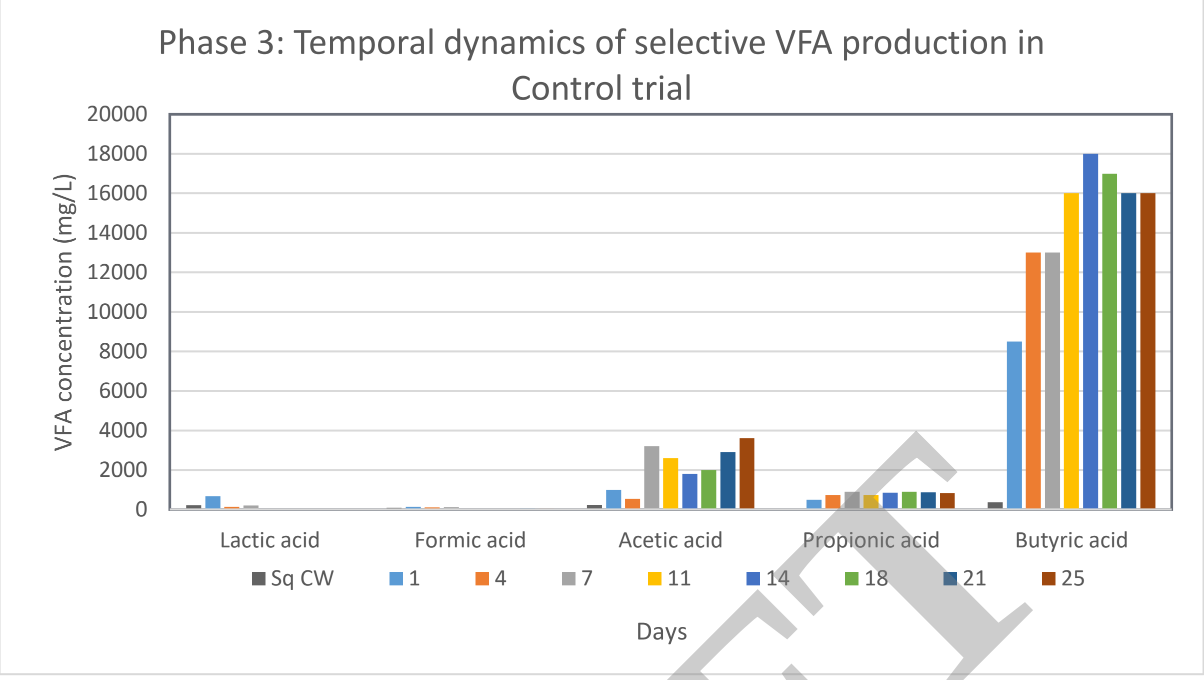

Figure 26. Selective VFA production over time during phase 3 - Control trial.

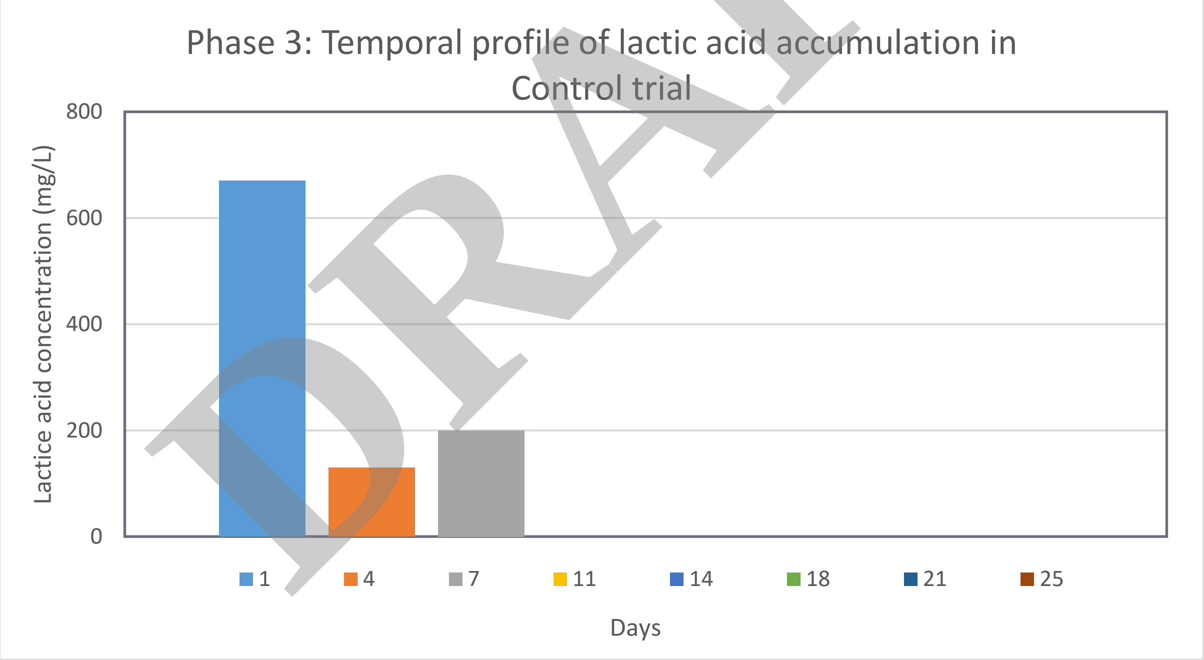

Figure 27. Lactic acid consumption over time during Phase 3- Control trial.

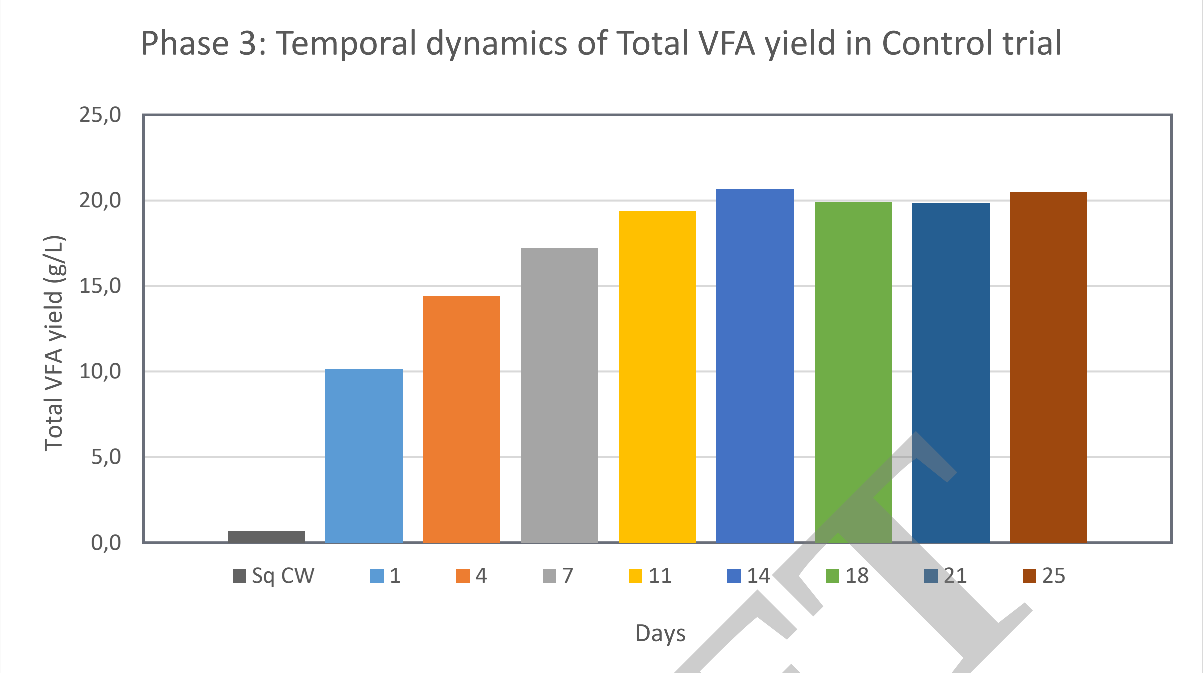

Figure 28. Total VFA yield over time during phase 3- Control trial.

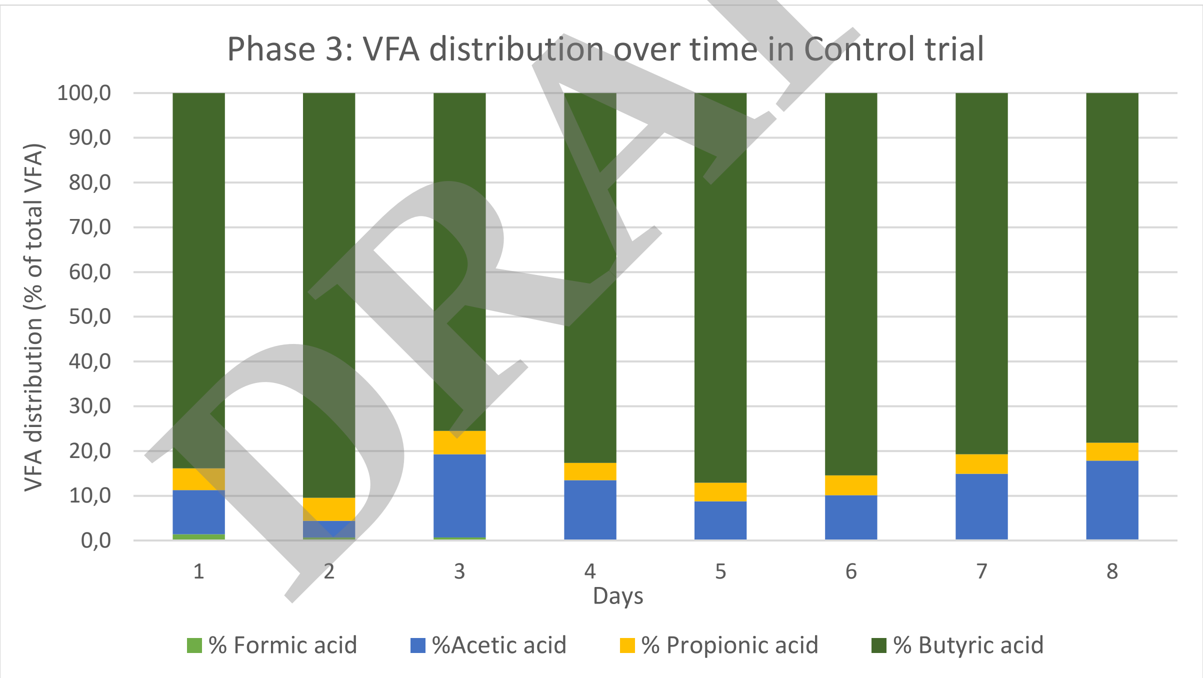

Figure 29. VFA distribution over time during phase 3- Control trial.

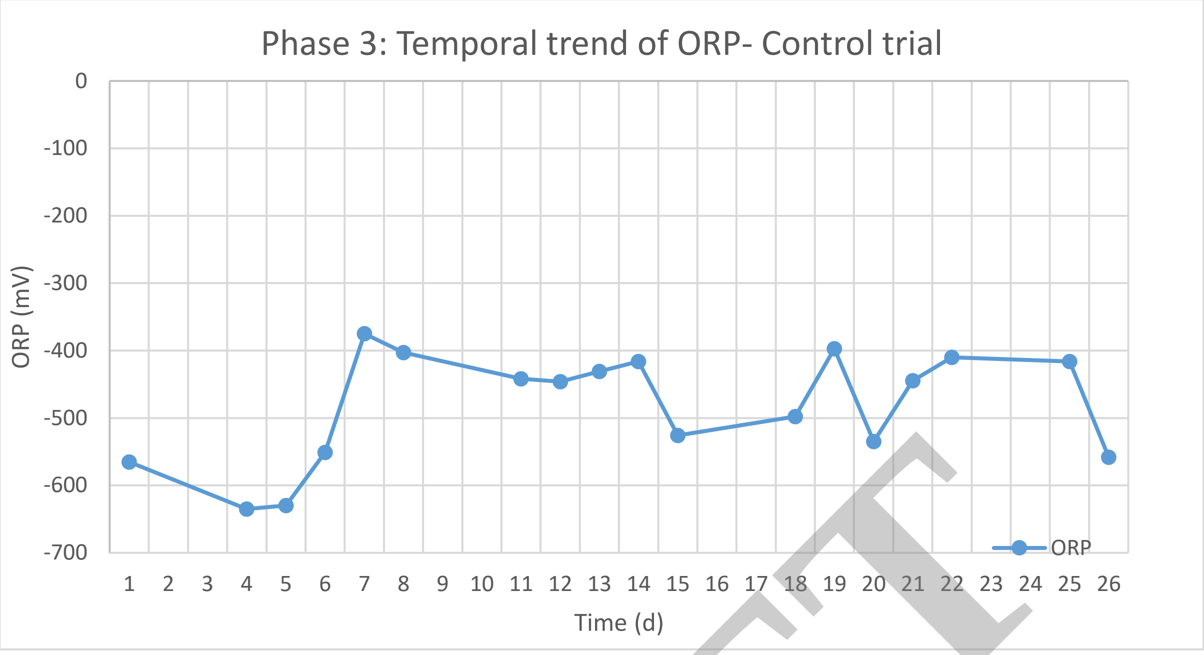

Figure 30 Temporal variation of ORP during phase 3- Control trial.

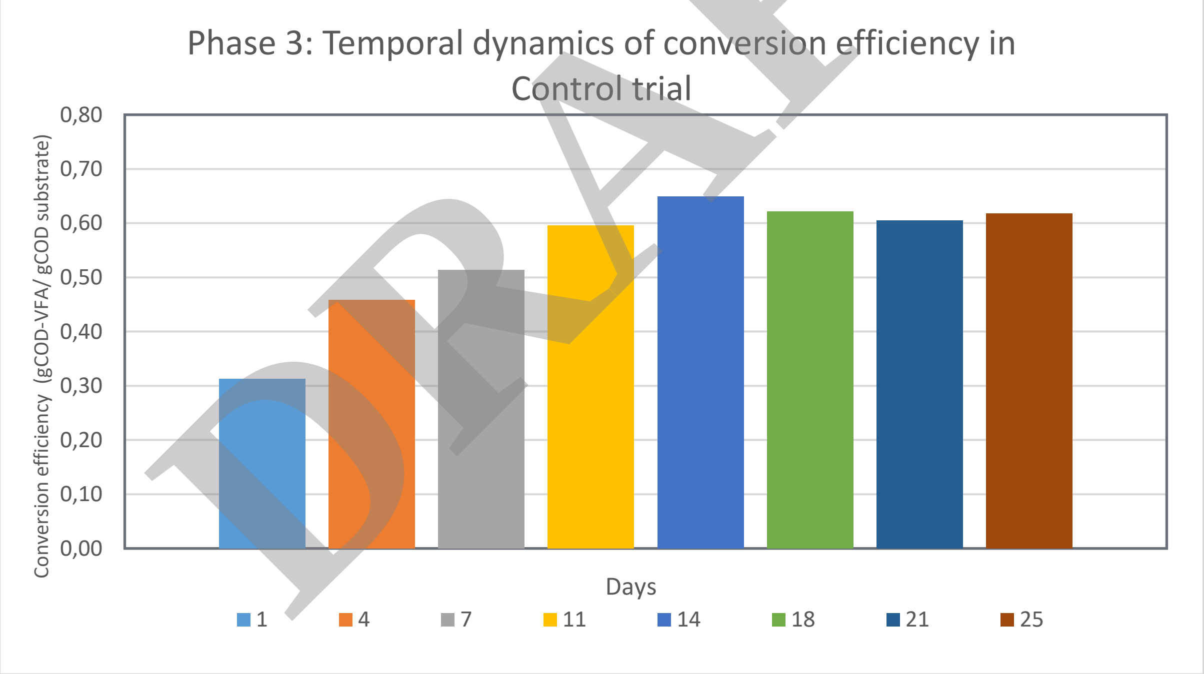

Figure 31. Process substrate conversion efficiency over time during phase 3- Control trial.

In the control trial of Phase 3, conducted without inoculum but under neutral pH (7) and a reduced SRT of 20 days, the endogenous microbiota demonstrated a robust capacity for VFA production, albeit with different dynamics compared to the inoculated system.

As illustrated in Figure 26, butyric acid emerged as the predominant product, with concentrations peaking at around 18000 mg/L by day 14 and remaining stable thereafter. Acetic acid followed with values up to 3500 mg/L, while propionic acid stayed consistently low (below 1000 mg/L), suggesting that neutral pH conditions alone were not sufficient to stimulate propionate production in the absence of *Propionibacterium*.

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The lactic acid profile (Figure 27) showed initial accumulation (670 mg/L on day 1), followed by a degradation phase, with levels declining to less than 200 mg/L at day 7 and total consumption from day 11 to end of test.

Total VFA yield (Figure 28) reached 20.7 g/L by day 14 and remained stable through day 25, representing a significant improvement over earlier phases and confirming that the native microbial community was able to establish a functional fermentative ecosystem under neutral conditions.

The VFA distribution (Figure 29) was largely dominated by butyric acid, accounting for 80–90% of the total VFA fraction throughout the experiment, with acetic and propionic acids making up the remaining share in relatively constant proportions. Notably, formic acid was virtually absent.

Substrate conversion efficiency (Figure 31) showed a steady upward trajectory, reaching 0.65 gCOD/gCOD by day 14 and remaining above 0.60 thereafter. While slightly lower than the peak efficiency recorded in the inoculated system (0.72), these results confirm that the system without inoculum was able to achieve a high and stable conversion performance.

Figure 30 displays the ORP profile of the control trial during Phase 3. The redox potential dropped sharply below –600 mV within the first few days, remaining consistently lower than in the inoculated counterpart. While anaerobic conditions were clearly maintained throughout the experiment, the ORP values exhibited greater fluctuations and more extreme negativity compared to the *Propionibacterium* trial. These oscillations, often reaching values as low as –650 mV, may reflect a less metabolically balanced system, possibly due to slower microbial activity and the absence of inoculum.

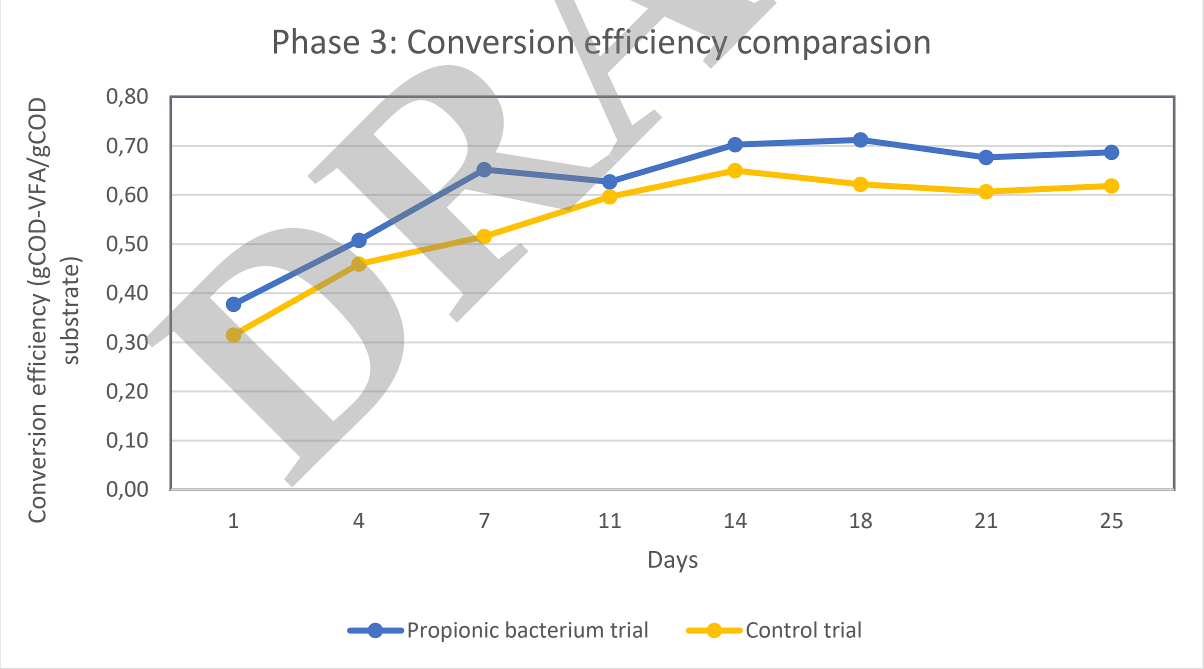

Figure 32. Comparison of substrate conversion efficiency during phase 3- Propionibacterium vs. Control trial.

The comparison of substrate conversion efficiency between the inoculated and non-inoculated systems during Phase 3 is presented in Figure 32. Both trials exhibited a steady increase in efficiency over time; however, the system inoculated with *Propionibacterium* consistently outperformed the control throughout the experimental period. In the early stages (days 1 to 7), the gap between the two conditions was more pronounced, with the inoculated trial reaching approximately

0.65 gCOD/gCOD by day 7, while the control lagged behind at around 0.52. This suggests a faster microbial adaptation and more efficient early-stage substrate utilisation in the presence of *Propionibacterium*. From day 11 onward, the control trial appeared to stabilise just above 0.60, whereas the inoculated system maintained a higher and more consistent performance, plateauing near 0.70–0.71 gCOD/gCOD. Although both systems ultimately reached stable operational regimes, the inoculated reactor demonstrated superior conversion efficiency across all timepoints, particularly in the initial phase. This difference indicates that targeted inoculation not only accelerates process start-up but also provides a measurable advantage in maximising the transformation of organic substrates into VFAs, even under neutral pH conditions and reduced SRT.

6.3.3 Phase 4

Phase 3 demonstrated that relatively shorter SRT (20 days) and controlled pH of 7 further improved process efficiency. However, it was considered to investigate the process potential for higher VFA yields at lower HRT. Thus, in Phase 4, an HRT of 6 days and SRT of 12 days were evaluated to assess their influence on process efficiency and the possibility of obtaining higher VFA throughput while reducing operation time. The process conditions, as well as results and discussion, are presented below.

Table 14. Process conditions of phase 4.

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37
SRTs	days	12
HRT	days	6
pH	-	7

Propionibacterium strains trial

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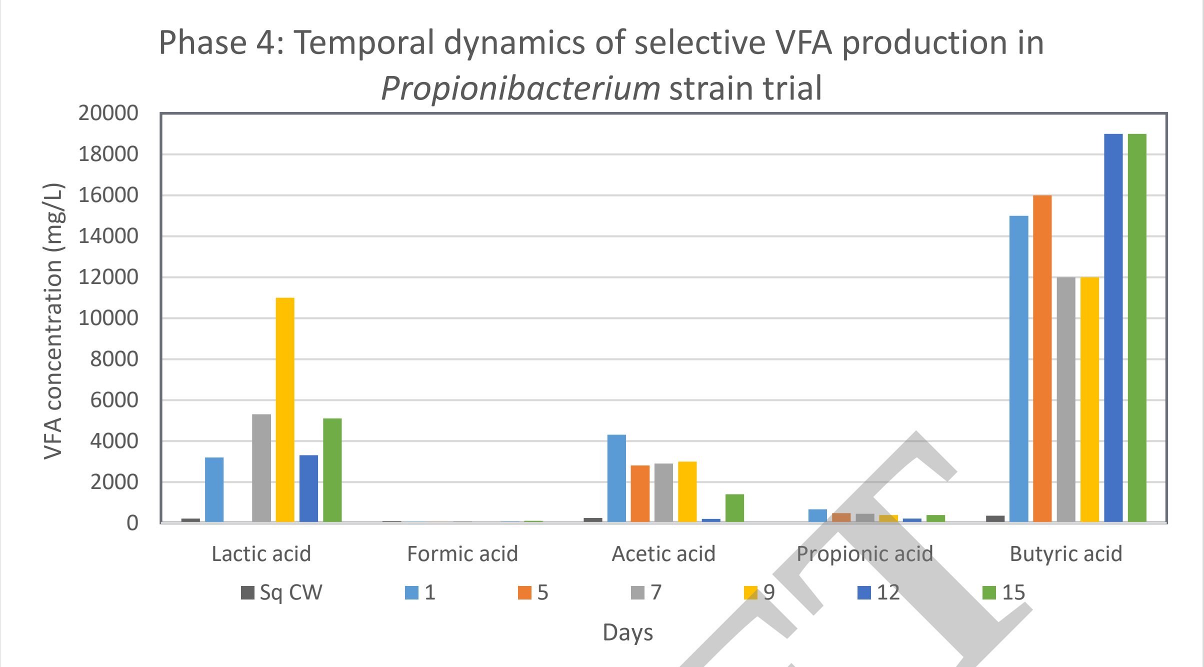

Figure 33. Selective VFA production over time during Phase 4- Propionibacterium trial.

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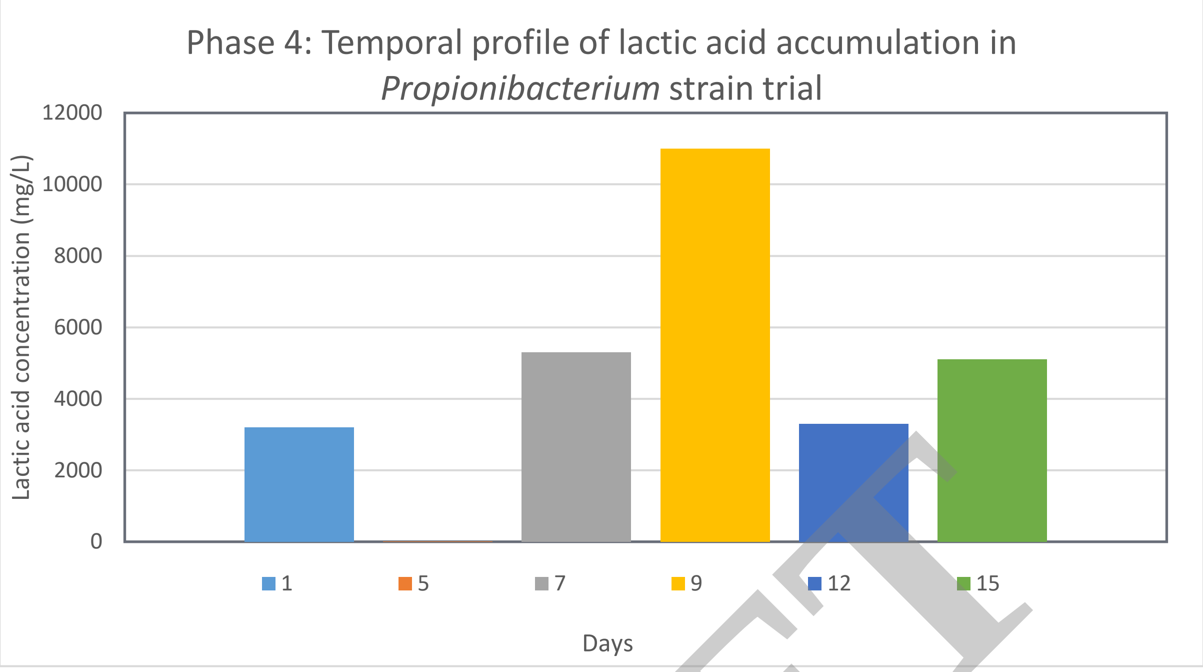

Figure 34. Lactic acid consumption over time during Phase 4- Propionibacterium trial.

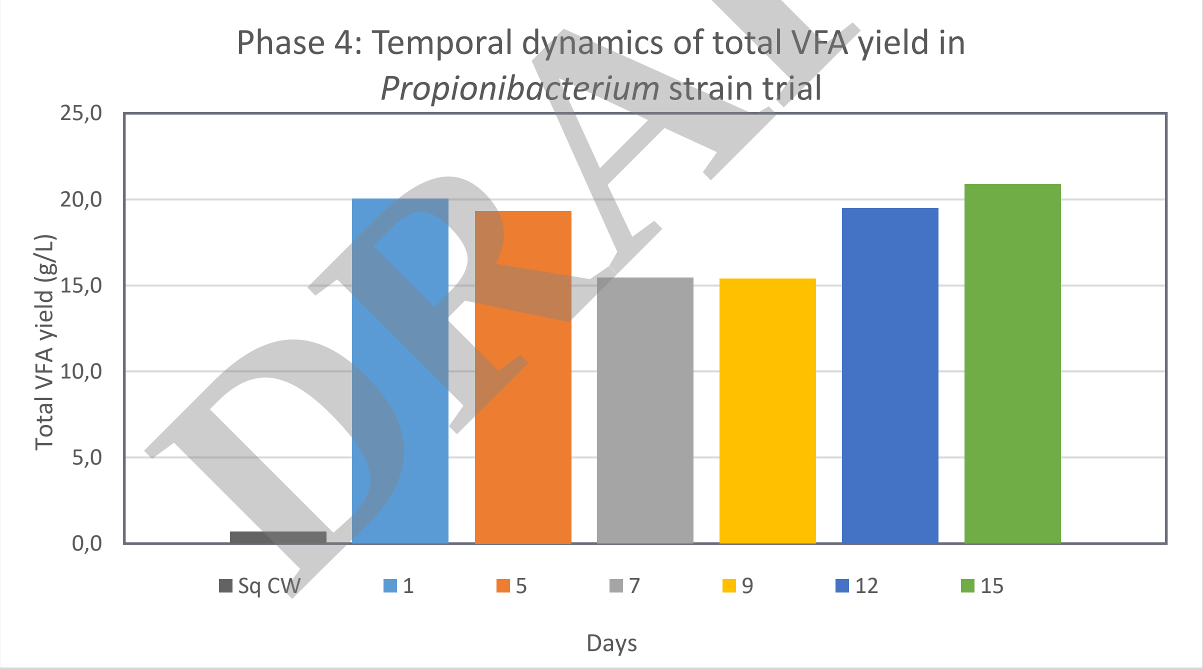

Figure 35 Total VFA yield over time during phase 4- Propionibacterium trial.

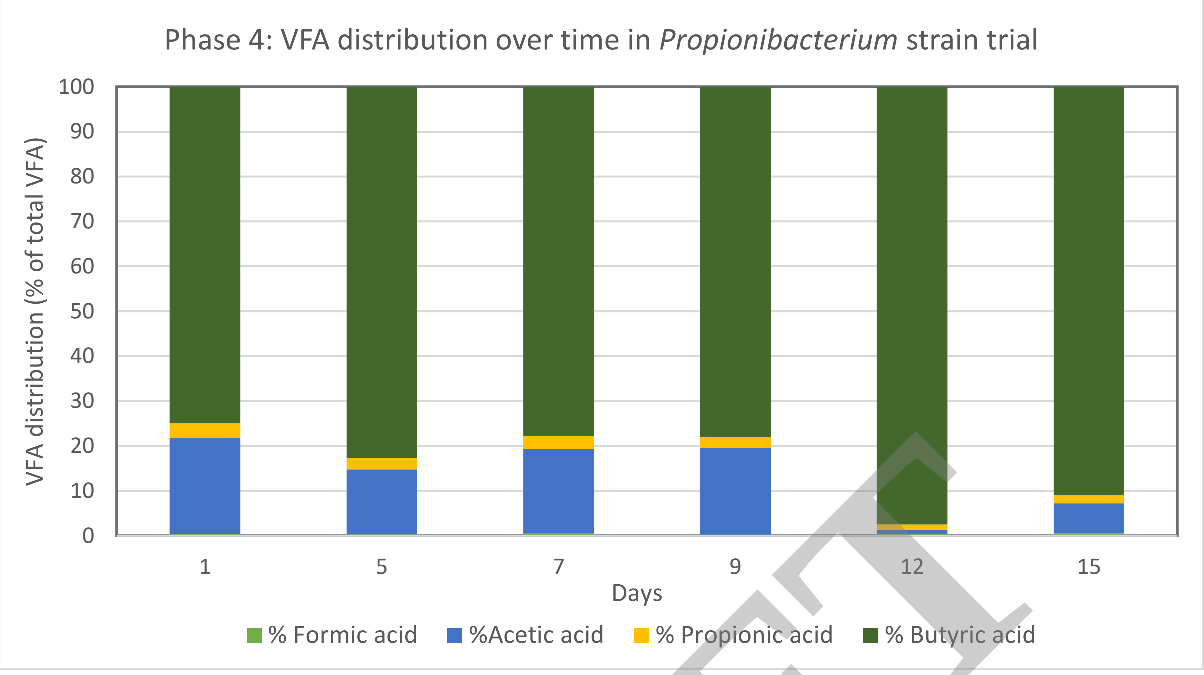

Figure 36. VFA distribution over time during phase 4- Propionibacterium trial.

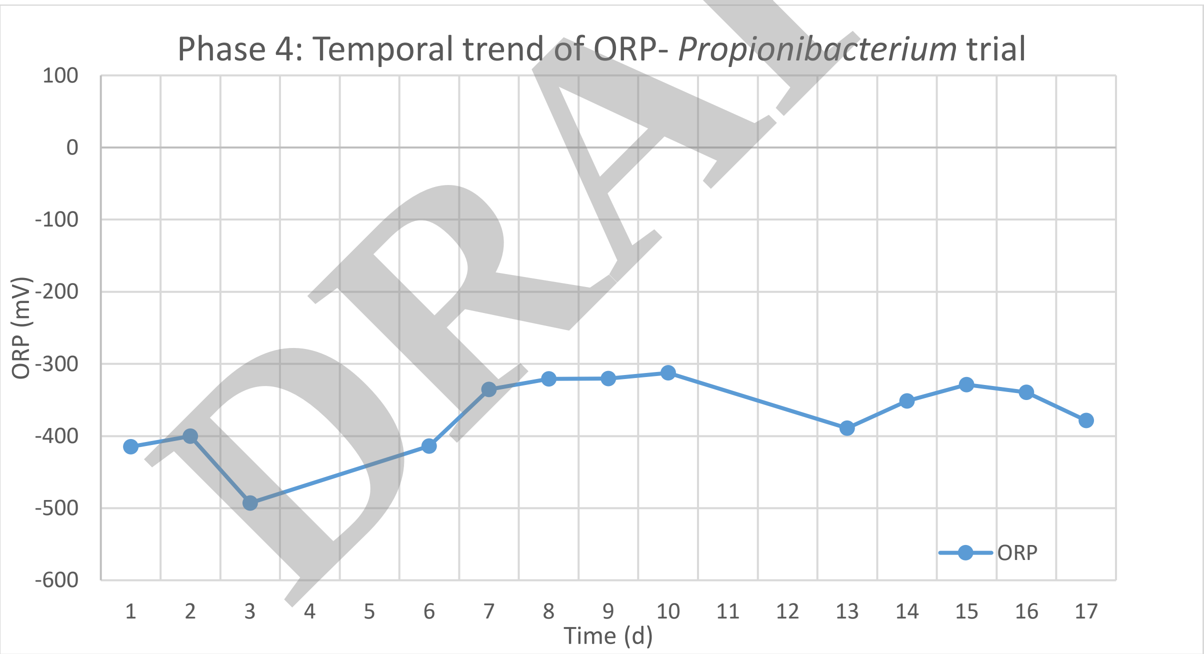

Figure 37. Temporal variation of ORP during phase 4- Propionibacterium trial.

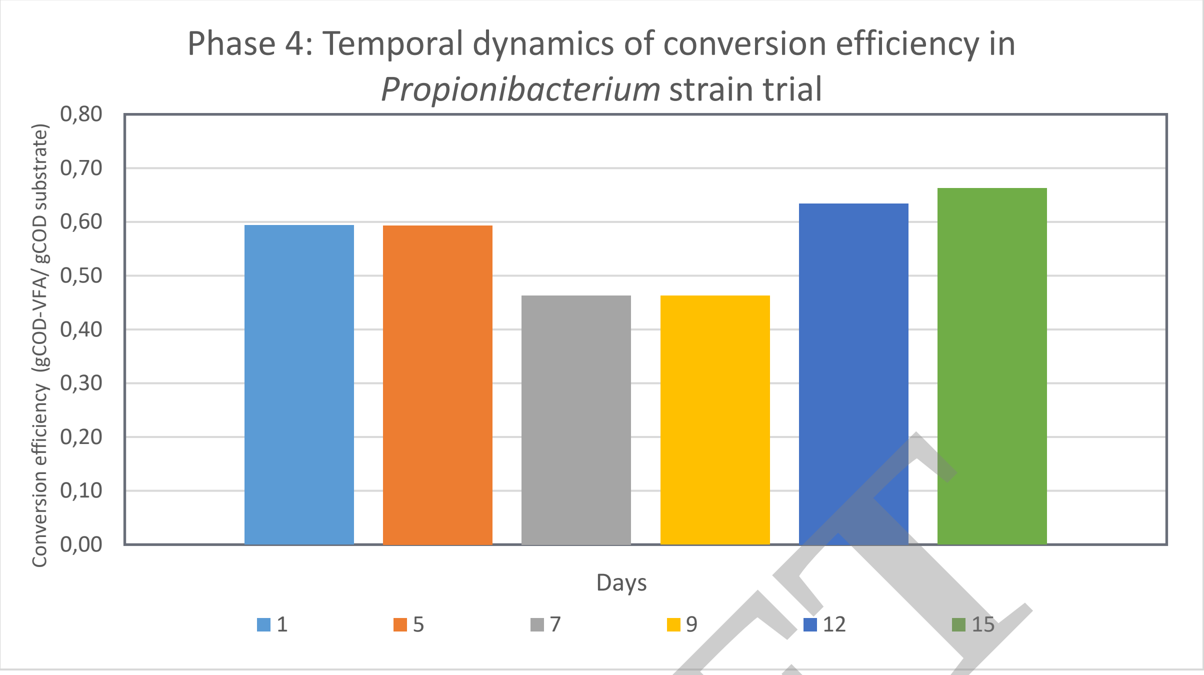

Figure 38. Process substrate conversion efficiency over time during phase 4- Propionibacterium trial.

The temporal dynamics of selective VFA production in the *Propionibacterium* strain trial (Figure 33) reveal distinct trends across different acids: lactic acid initially accumulates, with a sharp peak at day 9 (11000 mg/L), followed by a marked decrease by day 12, indicating its potential role as an intermediate that is rapidly consumed, likely by acidogenesis bacteria. Meanwhile, butyric acid steadily increases from the onset, surpassing 19000 mg/L by day 12, becoming the dominant VFA produced, and suggesting a metabolic shift favouring butyrate synthesis under the tested conditions. Acetic acid peaks early (day 1), followed by a gradual decline, while propionic acid reaches only modest concentrations, despite the presence of inoculum, suggesting a limited conversion efficiency or competitive metabolic routing.

This trend is corroborated by Figure 34, which isolates lactic acid and highlights its transient accumulation: after an initial build-up between days 1 and 9, the concentration drops significantly, confirming its subsequent consumption—likely by *Propionibacterium* and associated microbiota—as a preferred substrate.

Errore. L'origine riferimento non è stata trovata. presents the total VFA yield over time, showing high and relatively stable production (around 20–21 g/L), with a slight dip at days 7 and 9, potentially corresponding to a metabolic lag or partial redirection of carbon flux during the peak lactic acid phase.

The composition of the VFA pool is further elucidated in Figure 36, which displays a shift in distribution: butyric acid progressively dominates the profile, reaching over 90% of total VFAs by day 15, with a peak of 97.5% at day 12. This highlights a strong selectivity toward butyrate over time, with minimal contributions from propionic acid, despite the theoretical potential for its biosynthesis from lactate.

Finally, Figure 38 shows the conversion efficiency of the process, expressed as the ratio between gCOD of VFAs produced and gCOD of substrate. The profile displays an initial plateau (0.59 at days 1 and 5), a dip during mid-phase (days 7 and 9), and a recovery to over 0.66 by day 15. This pattern aligns with the metabolic dynamics described above: the initial adaptation and substrate uptake is followed by a temporary imbalance—likely associated with lactic acid accumulation—and then a recovery phase characterized by active conversion to VFAs, especially butyrate.

The inoculated reactor exhibited relatively stable ORP values throughout the 15-day operation, fluctuating moderately between –500 mV and –280 mV. After an initial decrease to around –500 mV (day 3), the system gradually recovered to a less negative range (–300 mV to –380 mV), stabilising in this range for the remainder of the trial. This trend suggests that anaerobic conditions were successfully maintained while allowing moderate metabolic activity, in line with the steady production of VFAs observed during this phase.

Control trial

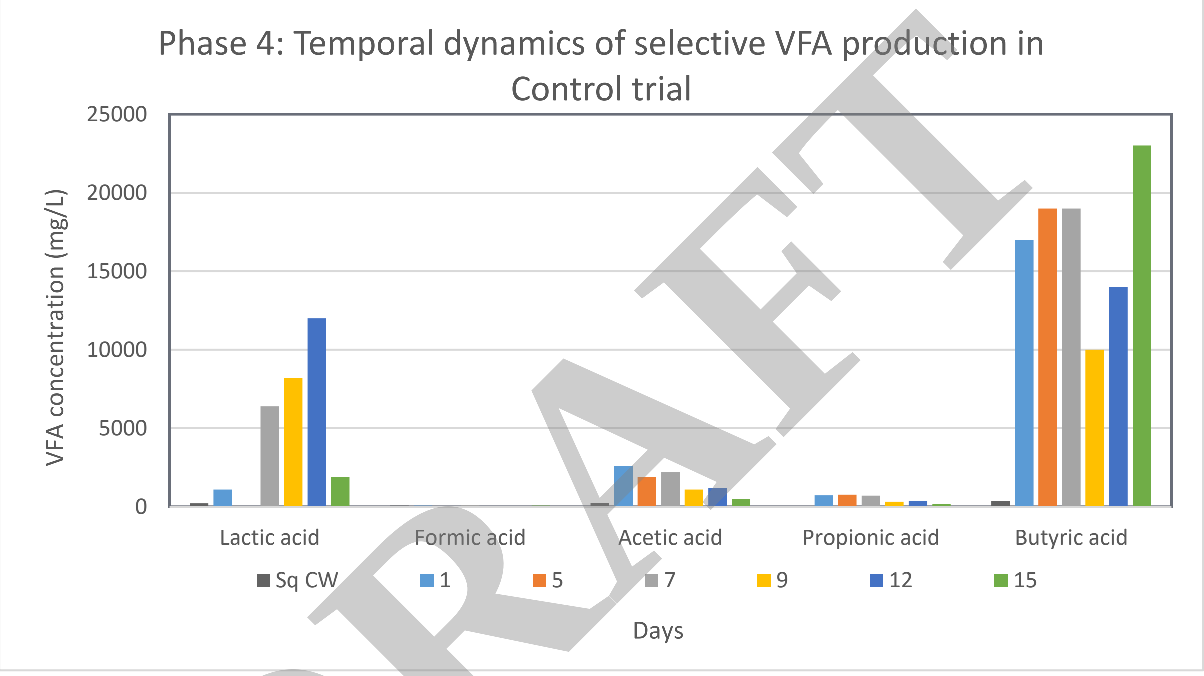

Figure 39. Selective VFA production over time during Phase 4- Control trial.

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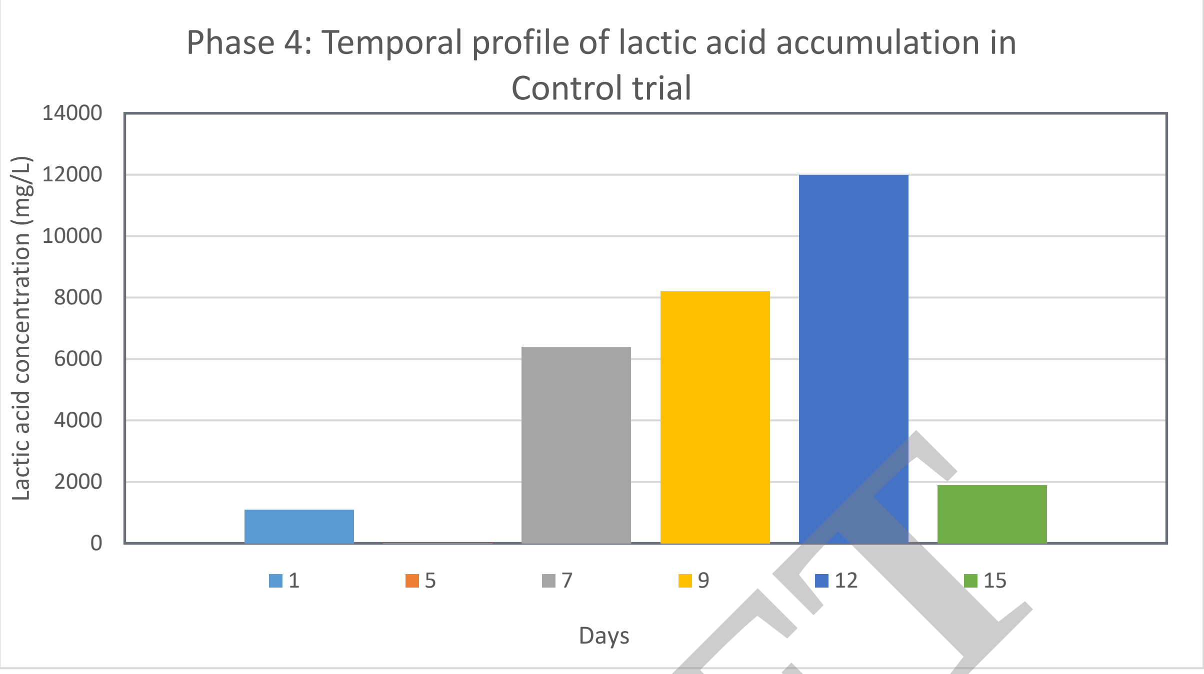

Figure 40. Lactic acid consumption over time during Phase 4- Control trial.

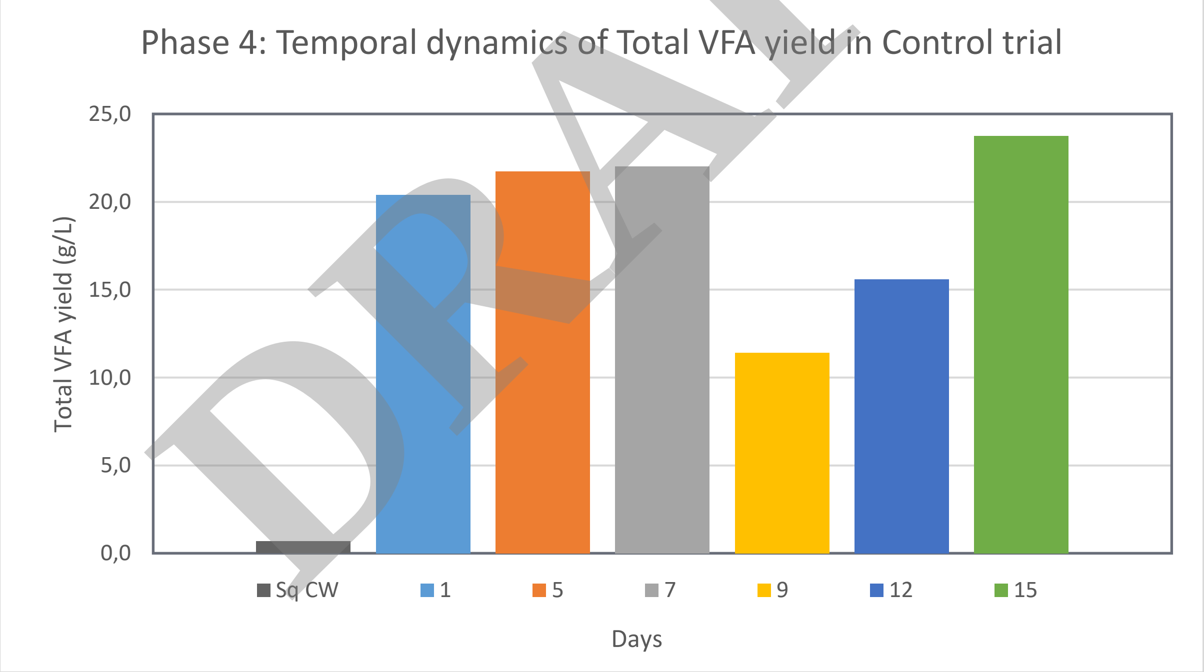

Figure 41. Total VFA yield over time during phase 4- Control trial.

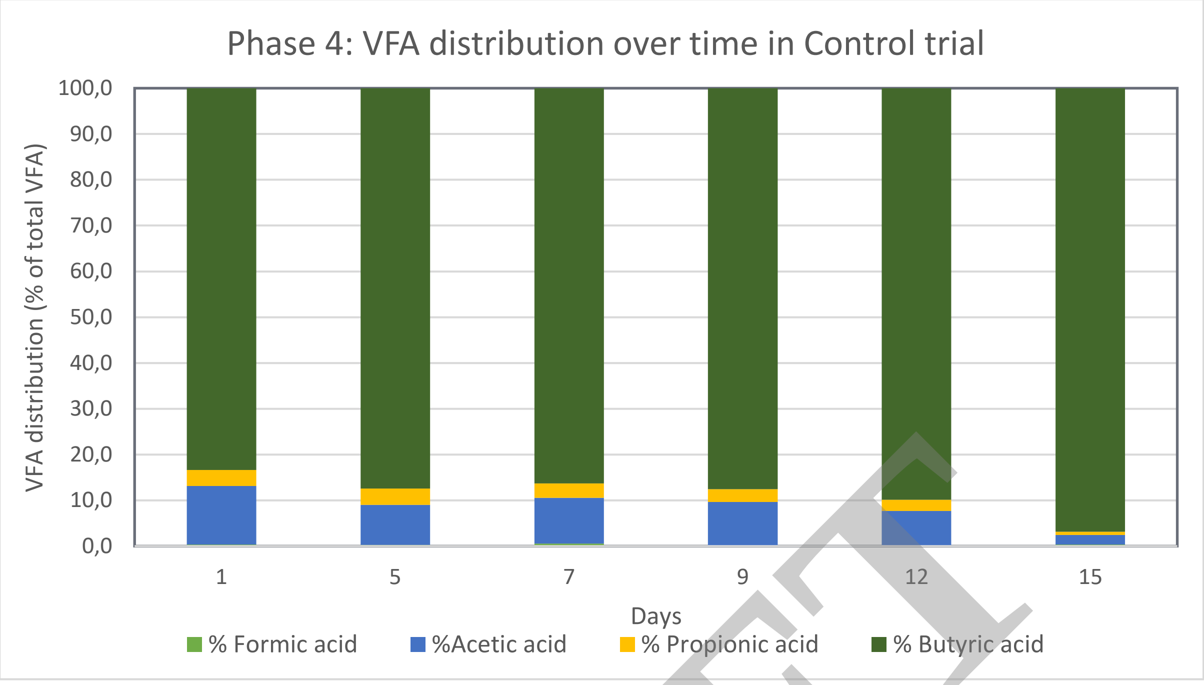

Figure 42. VFA distribution over time during phase 4- Control trial.

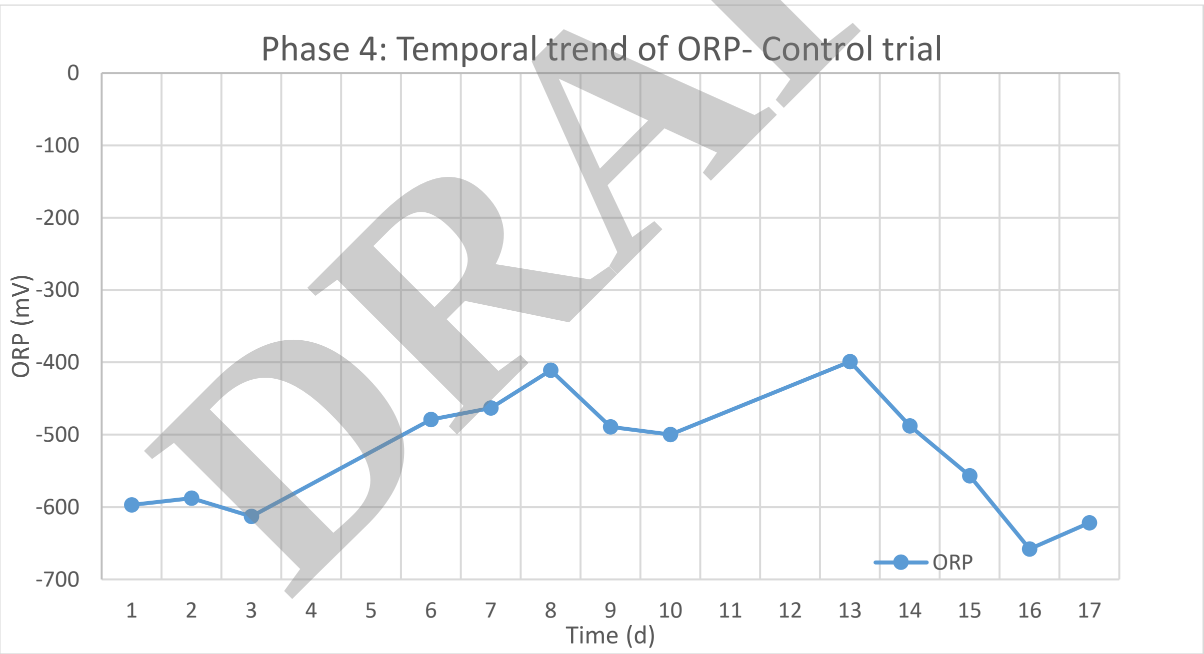

Figure 43. Temporal variation of ORP during phase 4- Control trial.

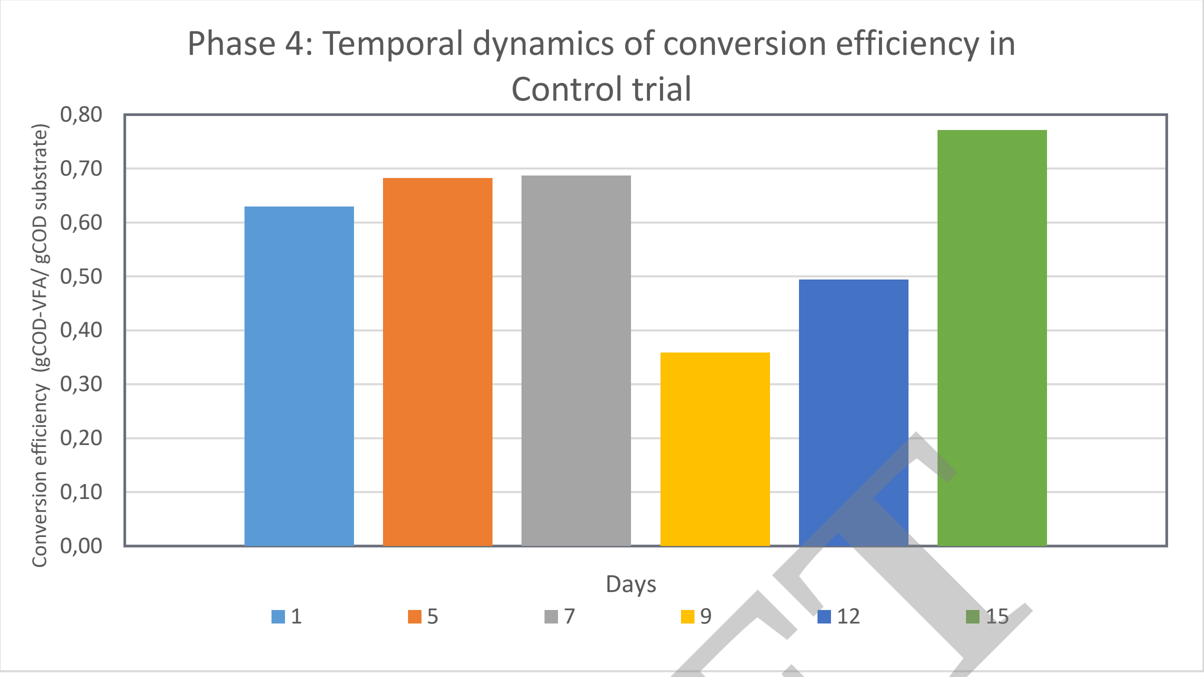

Figure 44. Process substrate conversion efficiency over time during phase 4- Control trial.

In the non-inoculated control trial conducted during Phase 4, the temporal dynamics of selective VFA production (Figure 39) revealed a clear predominance of butyric acid, which progressively increased over time, reaching its maximum concentration of approximately 23 g/L on day 15. This strong accumulation suggests that indigenous microbial communities were capable of sustaining butyrate-oriented fermentation even without external inoculum.

Lactic acid exhibited a characteristic accumulation–consumption pattern (Figure 41), with concentrations gradually increasing until day 12, peaking around 12 g/L, followed by a sharp drop on day 15, indicating a late-phase conversion of lactate—possibly into butyrate—through secondary fermentation pathways.

The total VFA yield profile (Figure 31) further confirmed the fermentative activity, with yields initially high (around 21–22 g/L by day 5–7), dropping to 12 g/L on day 9, and then recovering significantly, reaching the highest value of 23.8 g/L at day 15. This mid-phase drop may reflect a transient metabolic shift or substrate depletion, followed by resumed activity. The VFA distribution (Figure 42) consistently showed a highly selective profile dominated by butyric acid (>80%), while acetic, propionic, and formic acids remained minor components throughout, reinforcing the specificity of the metabolic routes activated under these conditions.

Finally, the conversion efficiency (Figure 44) closely mirrored the trends observed in total VFA production, with an initial stable performance (above 0.6 gCOD/gCOD) and a remarkable increase at day 15 (>0.75 gCOD/gCOD), highlighting the effective utilization of the substrate by the resident microbiota in the absence of any external microbial addition.

Regarding the ORP (Figure 43) the control trial showed significantly more negative ORP values compared to the inoculated reactor, especially during the early and late stages of the trial. ORP levels dropped below –600 mV in the first few days and again from day 15 onward, suggesting a strongly reducing environment. Such low ORP values may indicate microbial stress or the accumulation of reducing metabolites in the absence of an actively maintained microbial consortium. A short recovery (days 6–13) towards –400 mV was observed but not sustained, possibly reflecting transient metabolic activity.

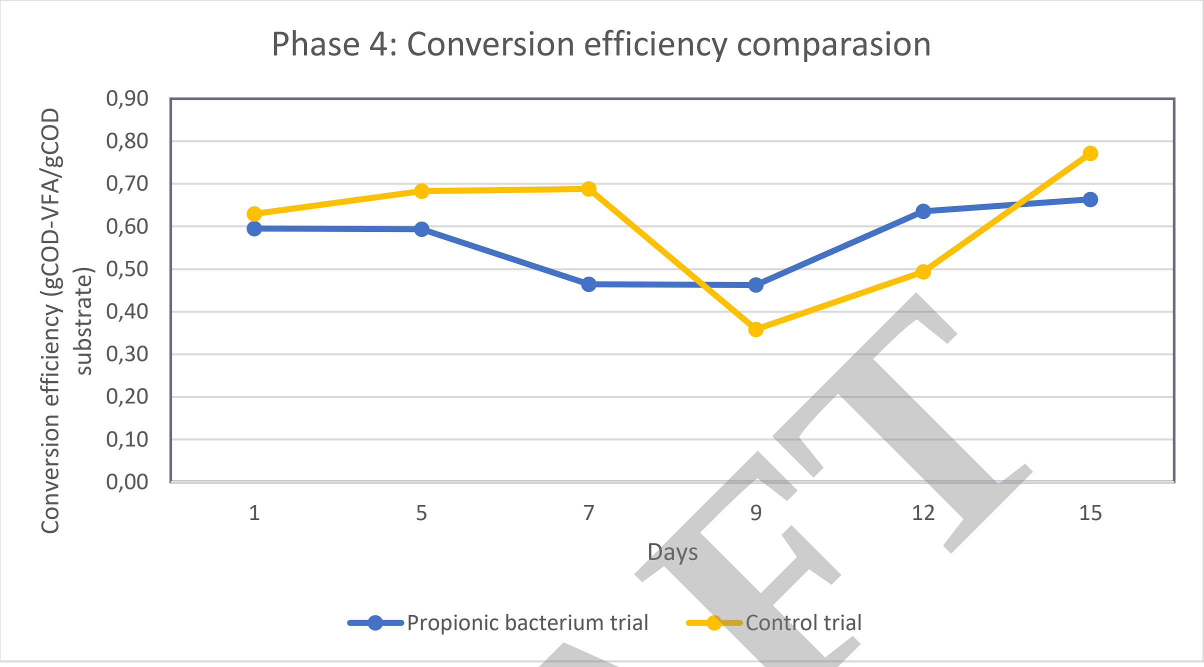

Figure 45. Comparison of substrate conversion efficiency during phase 4- Propionibacterium vs. Control trial.

A comparative analysis of substrate conversion efficiency during Phase 4, as depicted in Figure 45, highlights distinct temporal trends between the control trial and the inoculated system with *Propionibacterium*. Initially, the control trial demonstrated a slightly higher efficiency (0.65 gCOD-VFA/gCOD) than the inoculated counterpart (0.60 gCOD-VFA/gCOD), maintaining a stable and superior performance until day 7. However, after this point, the control trial underwent a marked drop, reaching its lowest efficiency of approximately 0.36 on day 9, likely due to substrate depletion or a temporary shift in microbial activity. In contrast, the *Propionibacterium* trial maintained a more stable conversion efficiency profile during the first half of the experiment, despite a slight decline until day 7, bottoming out at 0.46. Interestingly, the recovery phase between days 9 and 15 showed a sharp increase in both trials, with the control trial experiencing a more pronounced rebound, culminating in a final efficiency of 0.77, outperforming the inoculated trial, which peaked at 0.66.

6.3.4 Phase 5

In Phase 5, an HRT of 4 days was investigated, while the remaining parameters were similar to Phase 4. The aim was to investigate the influence of even shorter HRT on process efficiency and the possibility of obtaining higher VFA throughput. The process conditions, as well as results and discussion, are presented below.

Table 15 Process conditions of phase 5

Parameter	Unit	Value
Operation mode	-	Semi batch
Temperature	°C	37

SRTs	Days	12
HRT	Days	4
pH	-	7

Propionibacterium strains trial

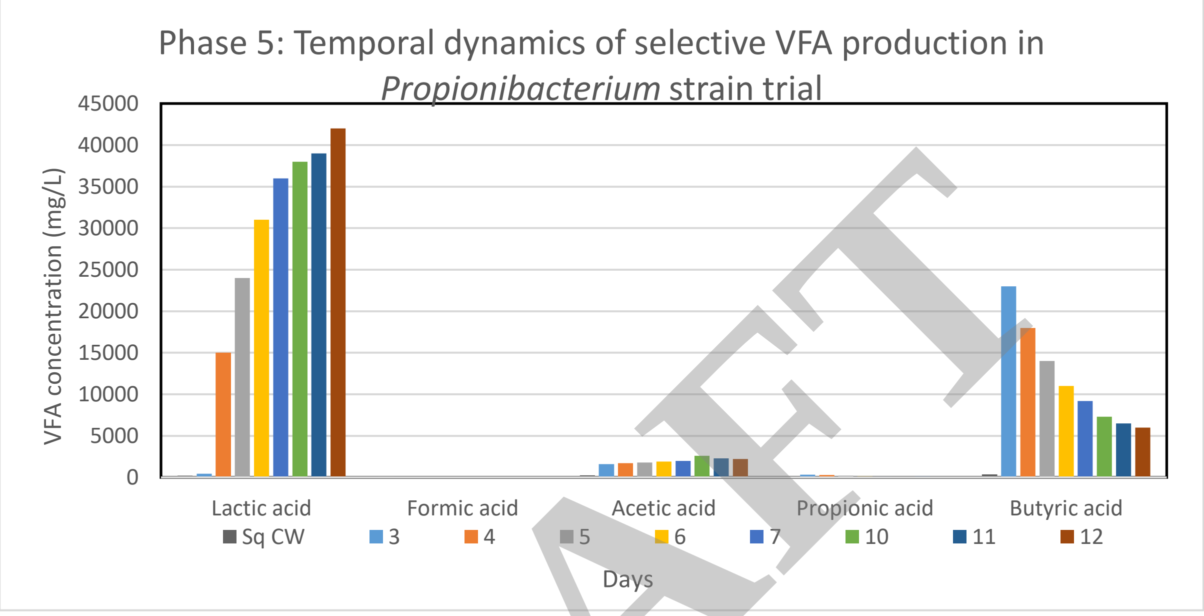

Figure 46 Selective VFA production over time during Phase 5- Propionibacterium trial.

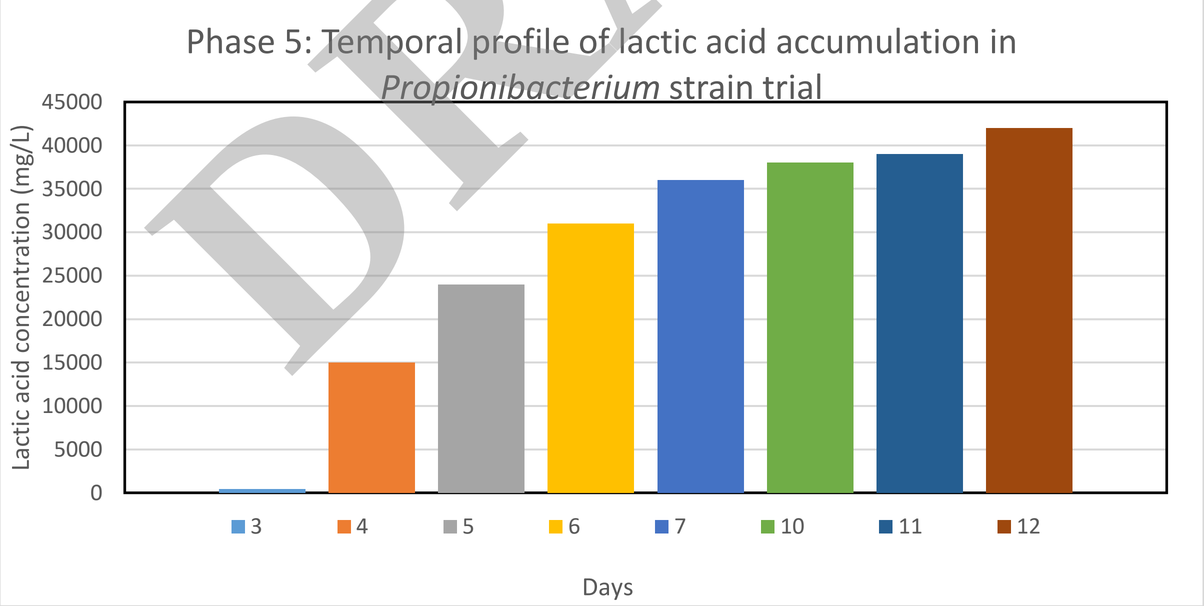

Figure 47 Lactic acid consumption over time during Phase 5- Propionibacterium trial.

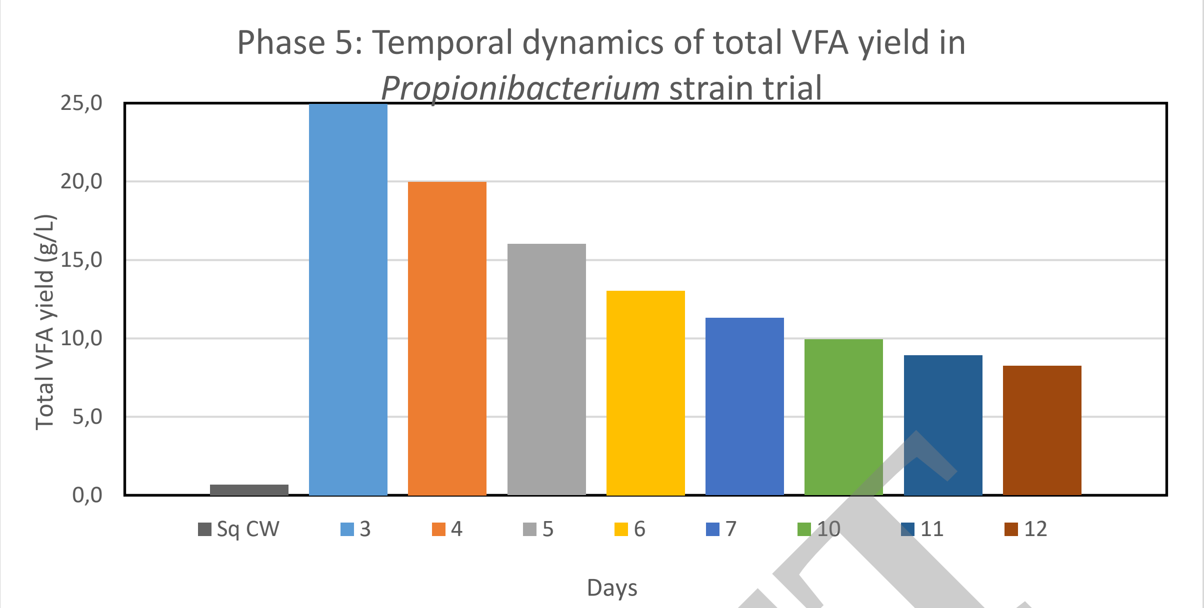

Figure 48 Total VFA yield over time during phase 5- Propionibacterium trial.

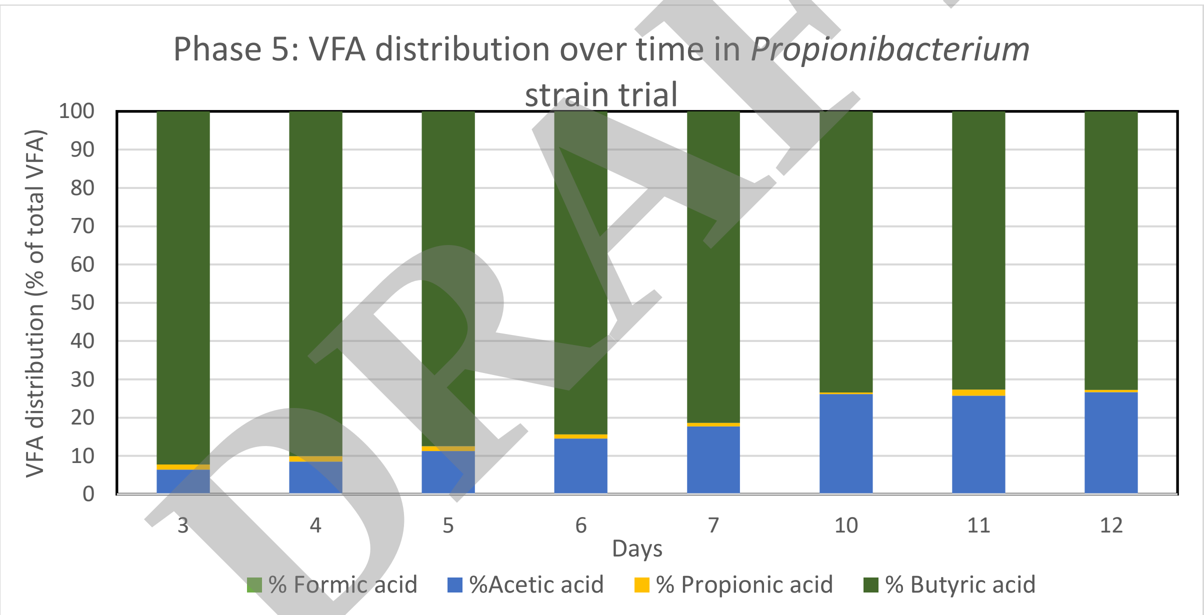

Figure 49. VFA distribution over time during phase 5- Propionibacterium trial.

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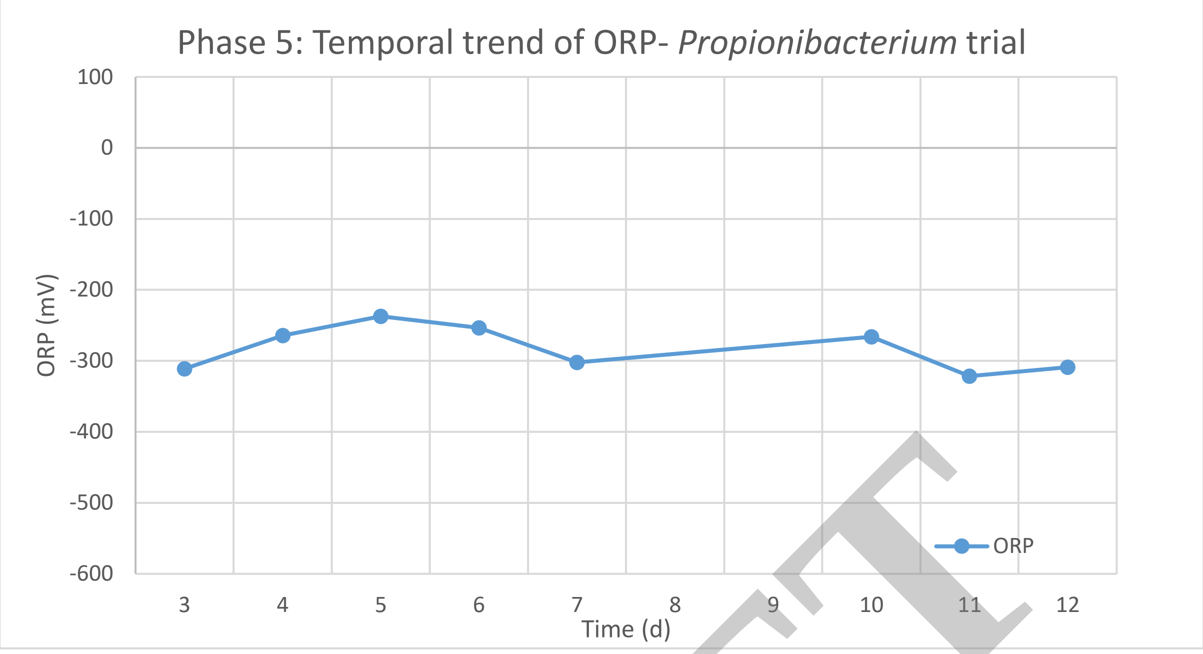

Figure 50. Temporal variation of ORP during phase 5- Propionibacterium trial.

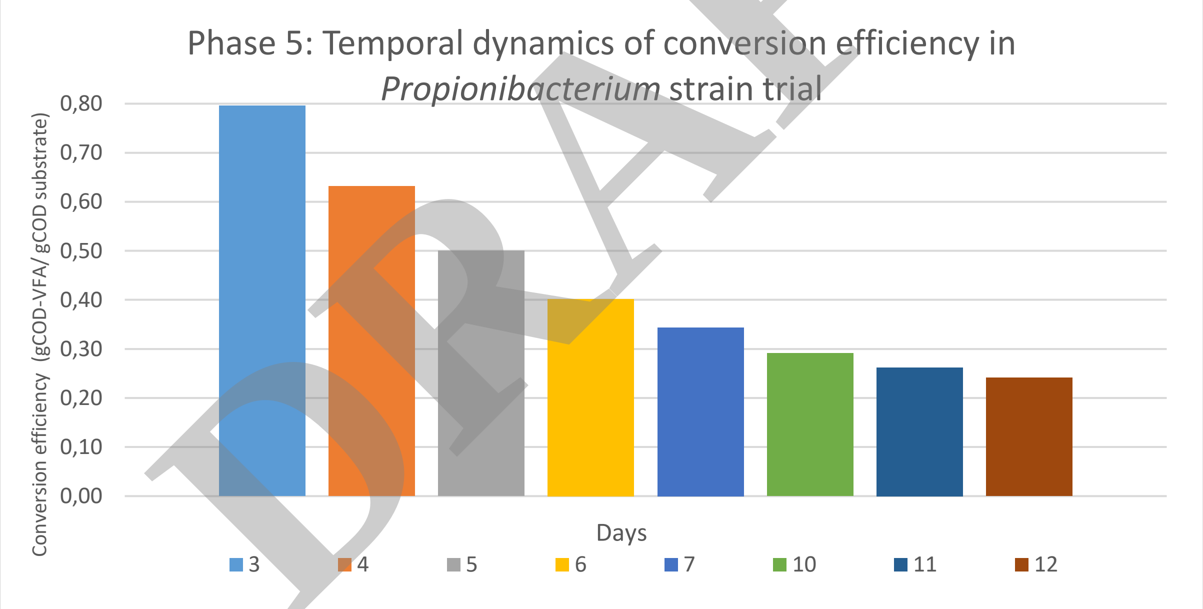

Figure 51 Process substrate conversion efficiency over time during phase 5- Propionibacterium trial.

In the *Propionibacterium* trial conducted during Phase 5, the temporal dynamics of selective VFA production (Figure 46) highlight a different metabolic profile. Lactic acid accumulated steadily and massively, reaching concentrations above 40000 mg/L by day 12, with limited conversion into downstream acids such as acetic and propionic acid, which remained below 2700 mg/L throughout. This is further illustrated in Figure 47, where the profile of lactic acid accumulation confirms a monotonic increase across all timepoints, indicating that the strain predominantly fermented sugars into lactate, but failed to further channel it into other VFAs. Consequently, as shown in Figure 48, the total VFA yield was highest at the earliest timepoint (day 3, >25 g/L) but declined progressively over time, likely due to lactic acid accumulation leading to feedback inhibition and reduced substrate conversion. The relative distribution of VFAs (Figure 49) further supports this, with butyric acid

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dominating at the beginning but progressively decreasing in favour of acetic acid, which peaked at around 25% of total VFAs by day 12. This shift suggests partial metabolic redirection, although the process remained largely dominated by non-converted lactic acid. Finally, Figure 39 shows the conversion efficiency trend (gCOD-VFA/gCOD substrate), which decreased over time from an initial value of 0.80 to a final value below 0.25, confirming a significant drop in fermentation efficiency under these conditions.

ORP values (Figure 50) remained relatively stable between –270 mV and –330 mV, indicating maintained anaerobic conditions. This controlled redox profile reflects the buffering role of the microbial consortium, though the lower VFA yield suggests metabolic limitations at low pH.

Control trial

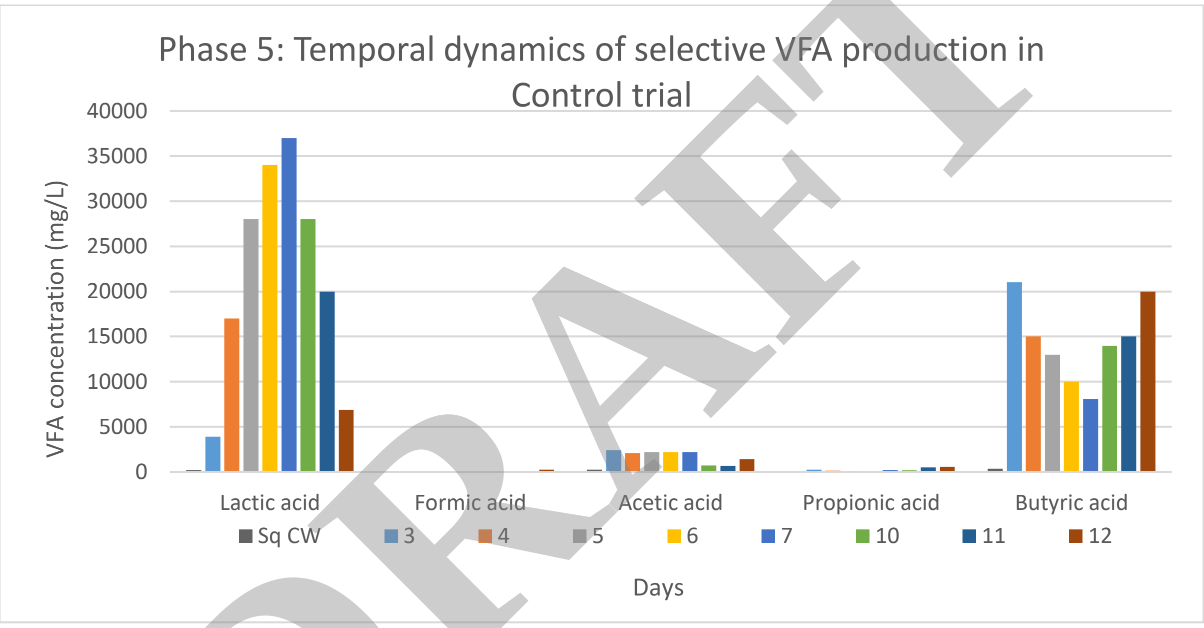

Figure 52 Selective VFA production over time during Phase 5- Control trial.

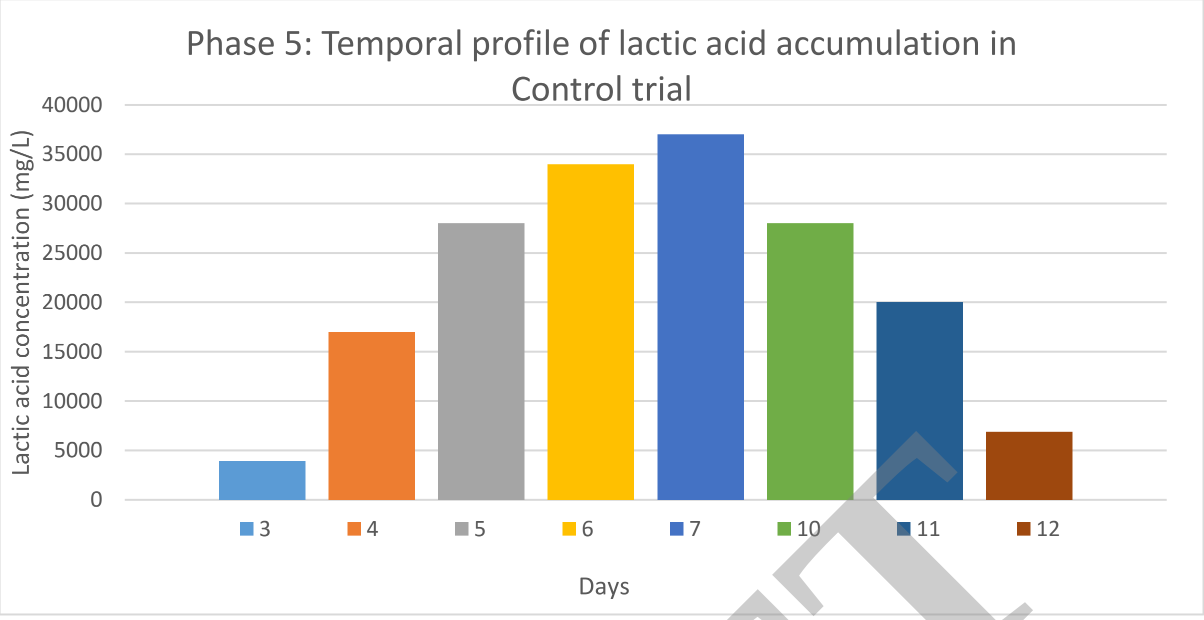

Figure 53 Lactic acid consumption over time during Phase 5- Control trial.

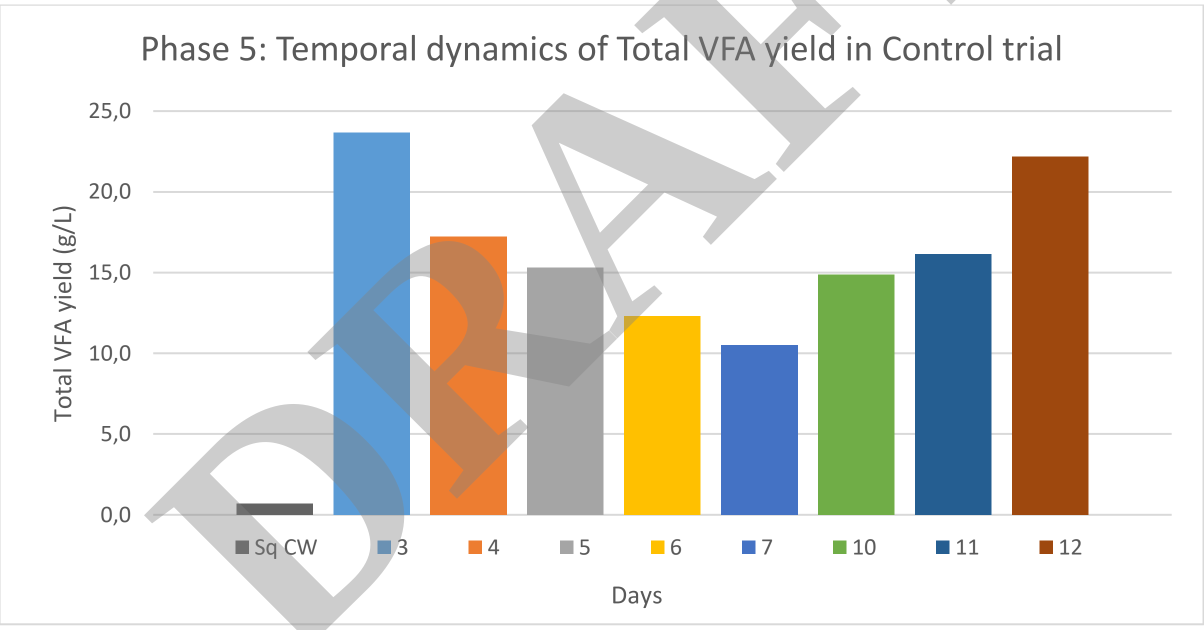

Figure 54 Total VFA yield over time during phase 5- Control trial.

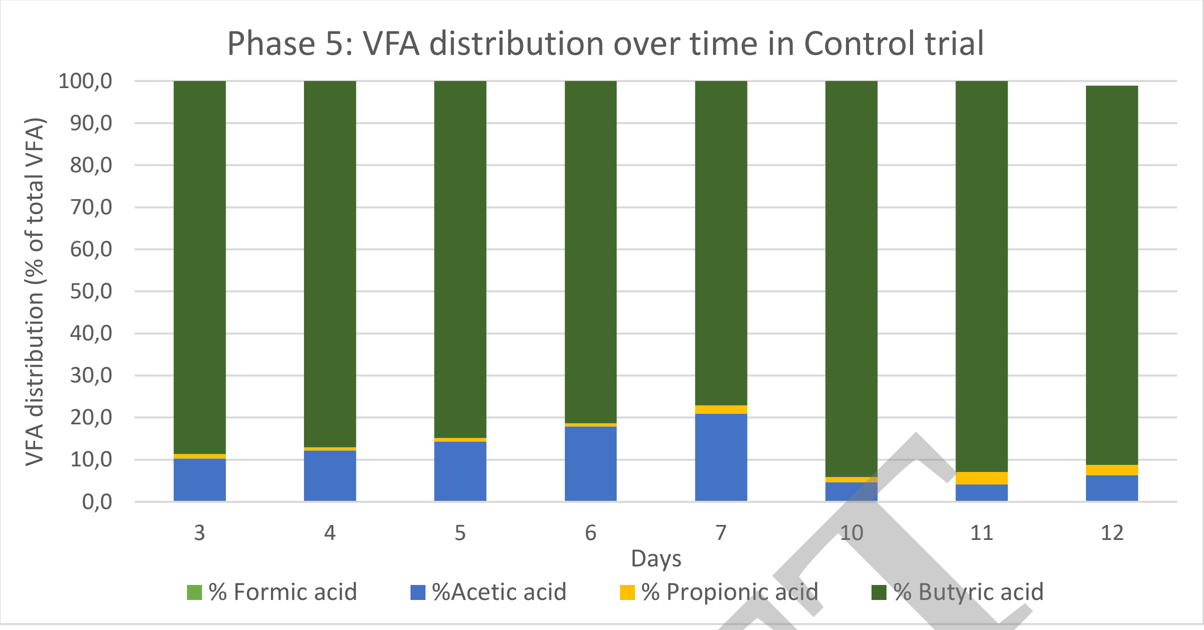

Figure 55 VFA distribution over time during phase 5- Control trial.

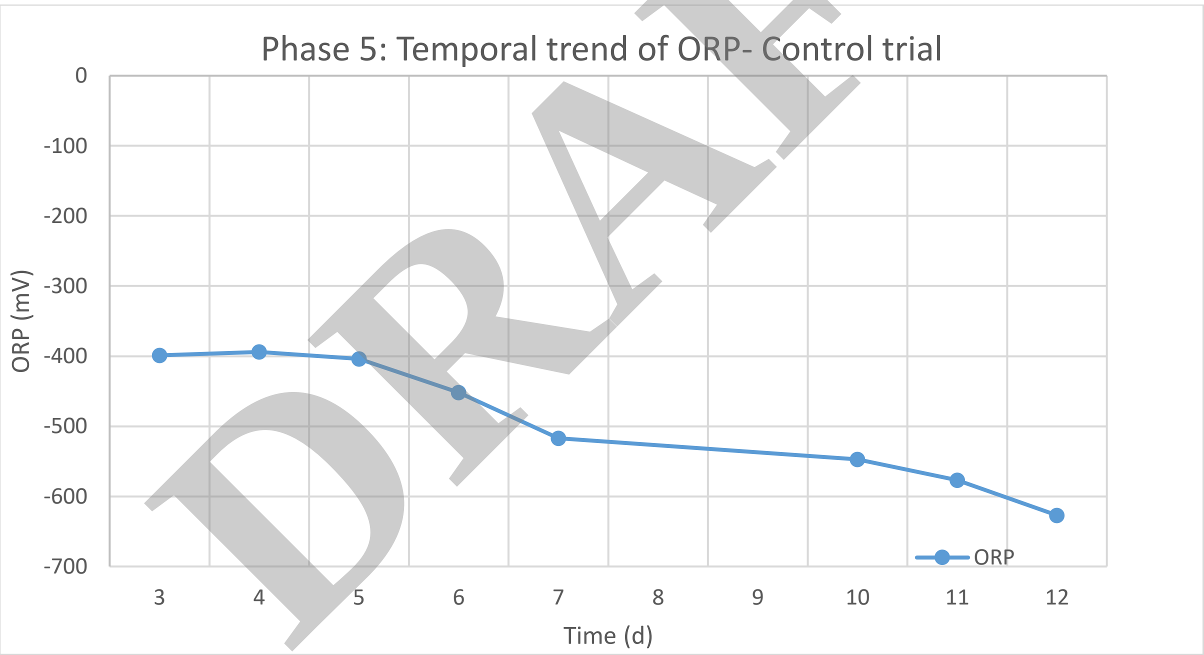

Figure 56. Temporal variation of ORP during phase 5- Control trial.

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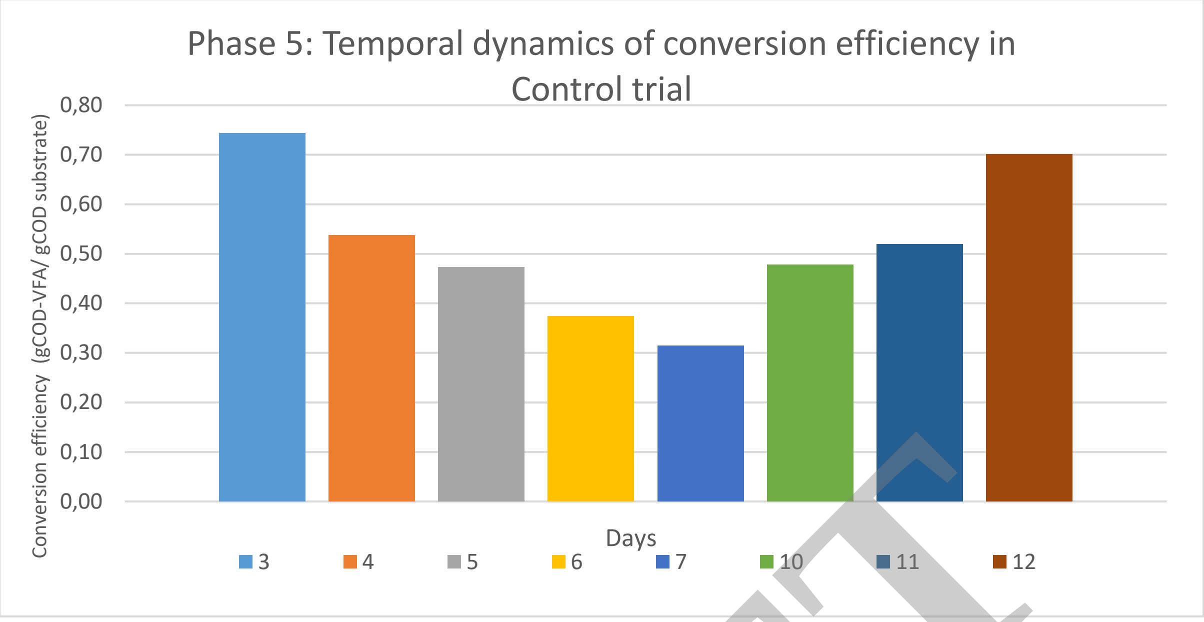


Figure 57 Process substrate conversion efficiency over time during phase 5- Control trial.

In the absence of inoculation, the temporal dynamics of VFA production during Phase 5 reveal a markedly different profile compared to the inoculated trial. Lactic acid accumulation dominates the early stages, rising steeply to a peak of approximately 37000 mg/L on day 7, as shown in both the total VFA production and lactic acid-specific plots. However, this peak is followed by a sharp decline to just over 6900 mg/L by day 12. This is further reflected in the overall selective VFA production trends, where butyric acid emerges as the second most prominent compound, maintaining concentrations between 8000 and 21000 mg/L, while acetic and formic acids remain in the low mg/L range and propionic acid is virtually absent. The temporal dynamics of total VFA yield show an initial maximum at day 3 (around 24 g/L), followed by a substantial drop around days 6–7, before partially recovering by day 12 (22 g/L). The acid distribution further highlights the predominance of butyric acid throughout the trial, consistently accounting for more than 70% of the VFA pool, with acetic acid contributing modestly and other acids remaining negligible. Finally, the conversion efficiency mirrors the trends seen in lactic acid accumulation and VFA yield, with an early drop from 0.75 to 0.31 gCOD-VFA/gCODsubstrate by day 7, followed by a steady recovery to 0.7 by day 12.

In the control trial (Figure 56), ORP steadily dropped to –670 mV, indicating excessively reducing conditions. Such extreme values may have negatively affected acidogenesis. The comparison between the results obtained from the inoculated and non-inoculated trials led to the conclusion that inoculation mitigated ORP drift, supporting process stability even under stress. These results underline the importance of redox and pH control for reliable VFA production in short-HRT regimes

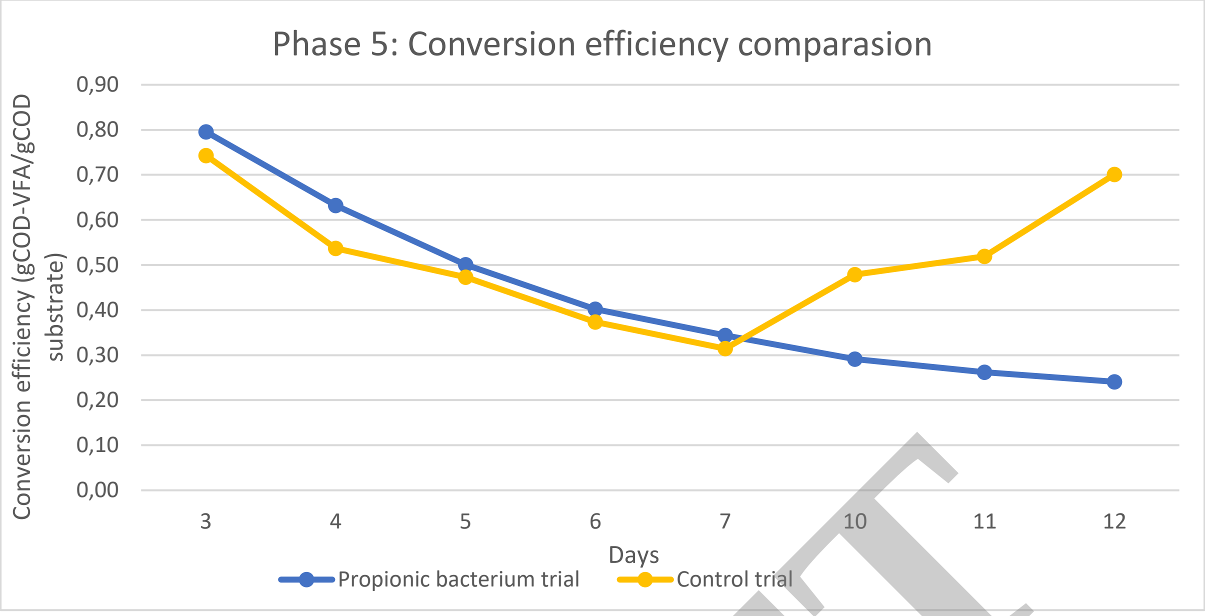


Figure 58 Comparison of substrate conversion efficiency during phase 5- Propionibacterium vs. Control trial.

The comparative analysis of conversion efficiency over time reveals distinct metabolic trajectories between the Propionibacterium-inoculated and control trials. Initially, both systems exhibit relatively high conversion efficiencies, with the inoculated setup starting at 0.80 gCOD-VFA/gCODsubstrate and the control at 0.73 on day 3. However, the inoculated system shows a continuous and steady decline in efficiency, reaching a low of 0.25 by day 12, suggesting a rapid substrate conversion in the early phase followed by possible inhibition or substrate depletion. In contrast, the control trial experiences a similar initial drop but demonstrates a marked recovery starting from day 7, reaching 0.70 by Day 12. This crossover trend indicates that while Propionibacterium accelerates early fermentation processes, native microbial consortia in the control condition progressively activate and sustain VFA conversion in the later stages.

Table 16. Overview of VFA Production and Operating Parameters Across Phases 1–5: Inoculated vs. Non-Inoculated Trials.

Phase n°	Process mode	Inoculated (yes or no)	HRT (d)	SRT (d)	Duration (d)	Inoculated			Control		
						pH (Avg±St.dv.)	Average Conversion efficiency (gCOD-VFA/gCOD-substrate)	Average VFA yield (g/L)	pH (Avg±St.dv.)	Average Conversion efficiency (gCOD-VFA/gCOD-substrate)	Average VFA yield (g/L)
1	Batch	No	10	10	10	3.60±0.36	0.032	0.873	3.60±0.36	0.032	0.873
2	Semi-batch	Yes	10	36	36	5.53±0.74	0.31	10.2	5.55±0.80	0.25	8.06
3	Semi-batch	Yes	10	20	25	6.54±0.39	0.62	20.2	6.38±0.45	0.55	18.8
4	Semi-batch	Yes	6	12	16	6.20±0.49	0.57	18.4	6.37±0.98	0.60	18.8
5	Semi-batch	Yes	4	12	12	5.64±0.25	0.43	14.1	5.36±0.30	0.52	15.5

Table 16 presents a comprehensive comparison of process parameters and VFA production outcomes across Phases 1 to 5, distinguishing between inoculated and non-inoculated (control) trials. In Phase 1, conducted in batch mode without inoculum and without pH control, the VFA yield was the lowest recorded (0.873 g/L). This result underscores the critical role of pH regulation in enhancing microbial activity and promoting VFA production. The average pH during this phase was approximately 3.6, a value insufficient to activate the microbial consortium effectively.

From Phase 2 onward, the introduction of inoculum under semi-batch conditions led to consistently higher VFA yields compared to the control, with the highest average yield (20.2 g/L) observed in Phase 3. This phase also registered the maximum peak VFA production (23.2 g/L), corresponding to the highest pH setpoint (approximately 6.5), confirming the positive effect of increasing pH on VFA productivity.

Throughout Phases 2 to 5, inoculated trials generally operated under optimized and often shorter hydraulic retention times (HRT) and solid retention times (SRT), while the control trials required equal or extended residence times to achieve comparable, though generally lower, yields. Despite a declining trend in average yield in Phases 4 and 5, inoculated systems consistently outperformed their non-inoculated counterparts, highlighting more efficient substrate conversion.

Importantly, results suggest that a pH of approximately 7 is optimal for VFA production. An HRT of 10 days emerged as the most favourable, while reducing HRT to 6 days resulted in only a modest 10% decrease in yield. However, a further reduction to 4 days led to a 44% decrease in VFA production, which may be partially attributed to an unintentional drop in pH to 5.6. Supporting this hypothesis, the comparison between Phase 2 (pH 5.5, yield 16.8 g/L) and Phase 5 (pH 5.6, yield 13.0 g/L) indicates that the reduction in HRT from 10 to 4 days alone accounts for an approximate 22% decrease in yield.

Overall, the data confirm the synergistic effect of inoculation and controlled pH in enhancing VFA production under semi-batch conditions, while also providing insights into the trade-offs associated with reduced retention times.

Furthermore, the relationship between ORP and Acetic acid was examined to see whether changes in ORP conditions in the reactor influence acetic acid production (Figure 59 to Figure 62). A certain correlation emerged in the control trial during phases 3 and 5. Still, its validity will be checked further in pilot-scale trials to confirm this hypothesis.

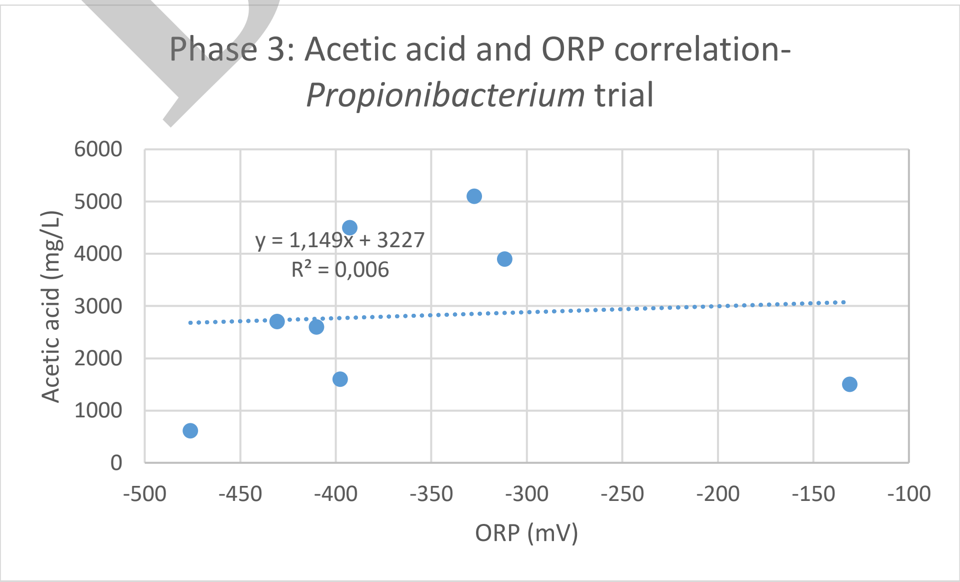


Figure 59 Phase 3: Acetic acid correlation with ORP conditions- Propionibacterium trial

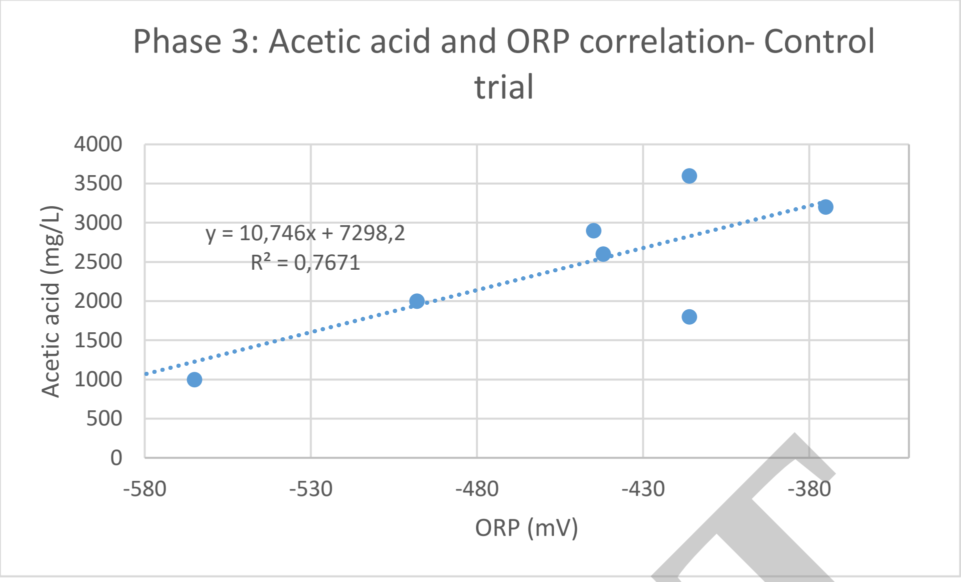


Figure 60 Phase 3: Acetic acid correlation with ORP conditions- Control trial

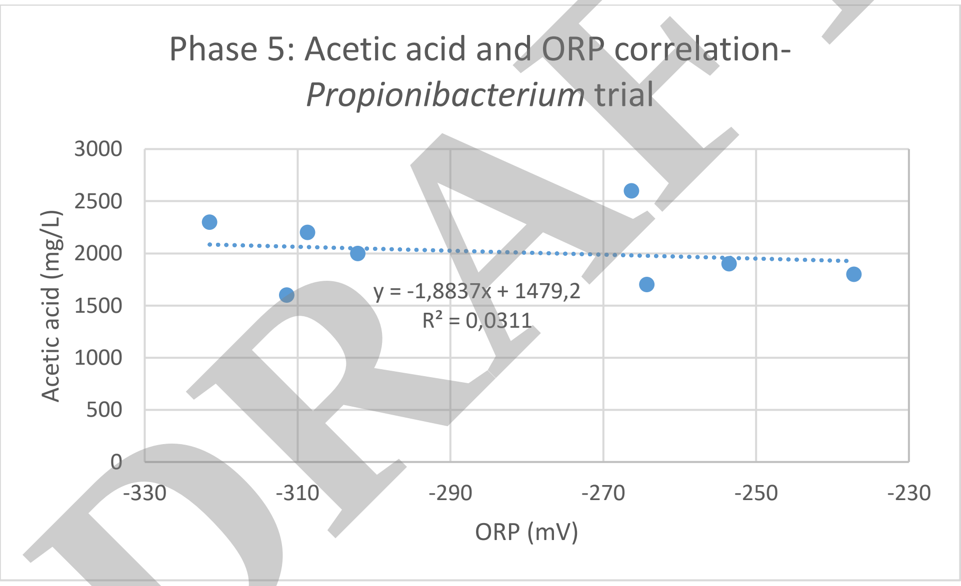


Figure 61 Phase 5: Acetic acid correlation with ORP conditions- Propionibacterium trial

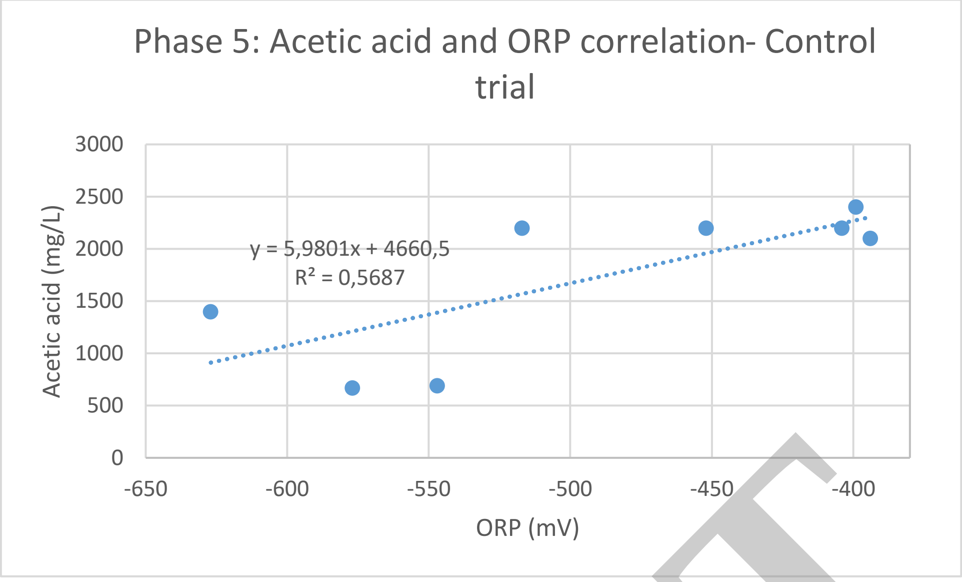
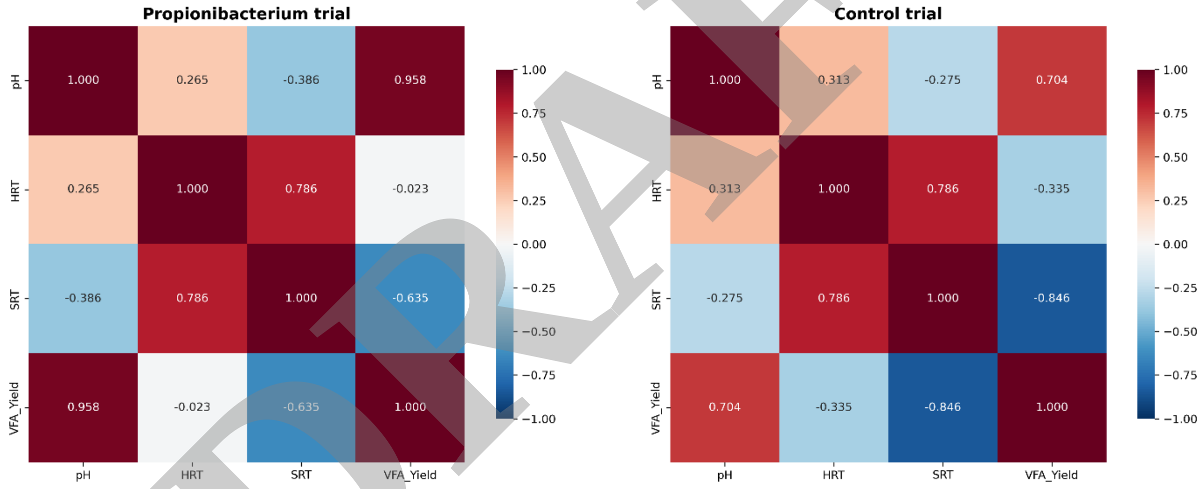


Figure 62 Phase 5: Acetic acid correlation with ORP conditions- Control trial



Propionibacterium trial

	pH	HRT	SRT	VFA_Yield
pH	1.000	0.265	-0.386	0.958
HRT	0.265	1.000	0.786	-0.023
SRT	-0.386	0.786	1.000	-0.635
VFA_Yield	0.958	-0.023	-0.635	1.000

Control trial

	pH	HRT	SRT	VFA_Yield
pH	1.000	0.313	-0.275	0.704
HRT	0.313	1.000	0.786	-0.335
SRT	-0.275	0.786	1.000	-0.846
VFA_Yield	0.704	-0.335	-0.846	1.000

Figure 63 Pearson correlation matrix of various parameters for both the Inoculated and Control trials

Errore. L'origine riferimento non è stata trovata. displays Pearson correlation matrices for key operational variables, pH, HRT, SRT and VFA yield in both the *Propionibacterium* inoculated and control trials.

In the *Propionibacterium* trial, a strong positive correlation was observed between pH and VFA yield (r = 0.958), highlighting the critical role of pH control in enhancing fermentation efficiency when the microbial inoculum is present. Conversely, VFA yield showed a moderate to strong negative correlation with SRT (r = –0.635), suggesting that extended biomass retention times may not favor VFA productivity under inoculated conditions. Interestingly, HRT had minimal correlation with VFA yield (r = –0.023), implying that substrate contact time was not the limiting factor in the inoculated system.

In the control trial, a similar positive correlation was found between pH and VFA yield (r = 0.704), though less pronounced than in the inoculated system, reinforcing the overall benefit of pH regulation.

However, SRT exhibited an even stronger negative correlation with VFA yield (r = –0.846), indicating that longer sludge retention significantly hindered production in the absence of the inoculum. Additionally, HRT showed a moderate negative correlation with VFA yield (r = –0.335), suggesting that longer hydraulic residence times were suboptimal for non-inoculated systems.

Overall, the matrices confirm that pH is a dominant driver of VFA yield, while excessive SRT may suppress production, particularly in non-inoculated conditions, and that inoculation not only enhances performance but also reduces system sensitivity to retention times.

6.4 Further Optimisation: from short trials to long-term pilot

Building on the results obtained across Phases 1 to 5, the upcoming optimisation will focus on consolidating the most promising operational strategies to enhance VFA productivity while ensuring long-term process stability and scalability. The comparative analysis clearly demonstrated that inoculation with *Propionibacterium* improved both average and maximum VFA yields, particularly under regulated pH conditions. Peak performance was observed in Phase 3, with an average VFA yield of 20.2 g/L and a maximum of 23.2 g/L, achieved under an HRT of 10 days, SRT of 20 days and pH maintained around 6.5. In contrast, control trials consistently yielded lower results, especially when the pH dropped below 6.0, confirming the critical role of pH regulation in supporting microbial activity and conversion efficiency, and high VFA throughput.

Reducing the HRT from 10 to 6 days (Phase 4) resulted in only a 10% decrease in yield, suggesting moderate process intensification is viable without substantial productivity losses. However, further shortening the HRT to 4 days (Phase 5) caused a sharper decline (–44%), likely due to uncontrolled pH drift to suboptimal values (5.6), rather than HRT alone. These findings highlight the importance of robust pH control as a cornerstone of the process strategy, particularly under intensified conditions. Correlation analyses further supported this, showing pH as the strongest positive predictor of VFA yield across both inoculated and control systems, while excessive SRT negatively impacted performance in the absence of active microbial enhancement.

To translate these insights into practice, the next optimisation step will involve the implementation of automated control strategies in a 500 L pilot-scale system. These will include:

(1) Continuous pH monitoring and adjustment to maintain the selected setpoint (6.5–7.0) via acid/base dosing.

(2) Automated nitrogen gas purging to preserve strict anaerobic conditions and prevent oxidative drift, if required.

(3) Programmable semi-batch feeding and withdrawal cycles, integrated with real-time sensor feedback (pH, ORP and conductivity).

(4) Validation of Phase 3-like conditions (optimal HRT/SRT/pH) under extended runtime to assess robustness, reproducibility, and scale-up performance.

(5) Once the feasibility of point 4 is validated in optimal results, the same operational conditions of phase 5 will be employed in the pilot scale system to assess whether the low VFA yield was due to pH drift or HRT itself.

A simplified control logic based on pH, coupled with ORP and conductivity data, as proxy indicators of metabolic activity, will also be developed to inform future automation strategies. The accumulated evidence underscores the importance of microbial inoculation, neutral pH maintenance, and carefully tuned retention times as the fundamental pillars of the production strategy.

6.5 Challenges encountered

During the initial development of the process, achieving stable anaerobic conditions proved challenging. Specifically, difficulties in reaching appropriate oxidation-reduction potential (ORP) levels necessitated nitrogen purging to remove dissolved oxygen from the reactor. This challenge was resolved by the end of Phase 1, allowing natural development of sufficiently reducing conditions without the need for nitrogen purging, thereby reducing both operational complexity and costs. In addition, challenges related to

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+-VFA recovery were addressed through a systematic evaluation of literature-based recovery techniques. Optimal recovery was achieved by adopting the method detailed in the section 5.3.3, which provided VFA recovery efficiency.

7 Main Findings and Conclusion

The experimental activities conducted within Subtask 2.1.1 successfully achieved the primary objective of establishing a laboratory-scale anaerobic fermentation process for the valorization of cheese whey into VFAs under mesophilic, semi-batch conditions. Across five progressive experimental phases, key operational parameters were systematically varied to optimize yield, and process stability. The main findings are summarized below:

- **Phase 1 – Batch, no inoculum, no pH control**: This initial trial yielded negligible VFA production (0.873 g/L), confirming that the absence of pH regulation (average pH 3.6) and inoculum critically limited microbial activity and acidogenesis. The result highlighted the need for both pH control and inoculum to initiate and sustain VFA production.
- **Phase 2 – Semi-batch, pH 6, with and without inoculum**: The introduction of pH control (setpoint 6.0) and inoculation with *Propionibacterium* markedly enhanced performance. The inoculated trial achieved 16.8 g/L of VFAs in 14 days, whereas the control reached 13.8 g/L only after 26 days. Butyric acid was the predominant component (65–87%).
- **Phase 3 – Semi-batch, pH 7**: This was the most productive condition overall. The inoculated trial yielded a maximum of 23.2 g/L and an average of 20.2 g/L VFAs, while the control reached 20.7 g/L. The VFA profile shifted toward greater butyric acid (up to 84%), increased acetic acid (11.5%), and reduced propionic acid (4%), confirming that neutral pH not only improves yields but also shapes the acid profile favorably.
- **Phase 4 – Semi-batch, pH 7, reduced HRT (6 d)**: Under moderate intensification, the inoculated trial still achieved high yields (20.9 g/L), while the control surprisingly reached 23.8 g/L. However, yield fluctuation was more pronounced, and VFA composition was strongly skewed toward butyric acid (>90%), with acetic acid ranging 2.1–6.7% and propionic acid remaining low (0.8–1.8%). This suggests reduced HRT affects system stability and selectivity.
- **Phase 5 – Semi-batch, uncontrolled pH (5.6), short HRT (4 d)**: Yield decreased substantially in both trials, particularly in the inoculated run (13.0 g/L), due to suboptimal pH. Despite the control trial reaching 22.2 g/L, correlation analysis suggests this was likely due to residual buffering rather than sustainable conversion. The loss in performance under pH drift confirms the essential role of maintaining neutral pH, especially when intensifying HRT.

Taken together, these results confirm that the optimal laboratory-scale conditions for cheese whey acidogenesis are:

- Neutral pH of 6.5–7.0, maintained via daily manual adjustment
- SRT between 12–20 days, depending on desired throughput
- HRT of 6–10 days, balancing productivity and process stability

These conditions enabled the highest and most reproducible VFA yields, fulfilling the objectives of Subtask 2.1.1 for production and recovery performance. Importantly, inoculated systems consistently outperformed controls in terms of yield, substrate conversion and metabolic stability, especially under pH-controlled regimes.

To support scale-up and integration with downstream valorization (e.g., PUFA production in Task 2.2), further trials will focus on validating these optimal conditions at pilot scale (T2.1.2, 500 L reactor). Continuous control strategies, including automated pH regulation and anaerobic integrity via nitrogen flushing, if needed, will be implemented to prevent process drift and enhance reliability. Additionally, Phase 5 highlighted the operational threshold for HRT reduction, showing that 4-day HRTs may compromise performance unless pH is rigorously controlled.

Overall, these findings demonstrate the feasibility of process intensification and inoculation-based enhancement for VFA recovery from cheese whey, providing a robust foundation for transition to TRL 5.

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