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Assessment of Protein-Rich Cheese Whey Waste Stream as a Nutrients Source for Low-Cost Mixed Microbial PHA Production

Catarina S. S. Oliveira *, Marisa O. D. Silva, Carlos E. Silva, Gilda Carvalho † and Maria A. M. Reis

UCIBIO-REQUIMTE, Dep. Chemistry, Faculty of Science and Technology, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal; amarisadasilva@gmail.com (M.O.D.S.); carloseduardosilva1989@gmail.com (C.E.S.); gs.carvalho@fct.unl.pt (G.C.); amr@fct.unl.pt (M.A.M.R.)

* Correspondence: css.oliveira@fct.unl.pt; Tel.: +351-212-948-300

† Current address: Advanced Water Management Centre (AWMC), The University of Queensland, St Lucia, QLD 4072, Australia.

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Abstract: The critical step in the mixed microbial cultures (MMC) polyhydroxyalkanoates (PHA) production process is the selection of an MMC enriched in an efficient PHA-accumulating organism, usually requiring growth-nutrients supplementation. This study aimed at assessing cheese whey (CW) proteins as a source of nitrogen for PHA-producing MMC, thus eliminating or reducing the need for nutrients supplementation. The selection reactor, operated with fermented CW, under a feast–famine regime, was initially supplemented with ammonia–nitrogen in a C/N ratio of 100/15 (Cmol/Nmmol), which was gradually reduced until eliminated, in order to select a culture capable of using CW proteins nitrogen. Decreasing nitrogen supplementation from a C/N ratio of 100/10 to 100/7.5 lead to significant microbial community changes, and reduced the MMC PHA-storing capacity, storage yield, and PHA productivity, decreasing by 30%, and over 45%, respectively. The PHA-storing capacity further deteriorated as nitrogen supplementation was reduced, despite increased protein-uptake. Results show that a culture enriched in PHA-accumulators capable of using proteins as sole nitrogen source could not be attained. In conclusion, this work reports for the first time that an easily bioavailable nitrogen source is required for efficiently selecting PHA-accumulating cultures. Based on the results obtained from this work, a pilot scale plant (two reactors of 100 L) fed with cheese whey for production of PHA is currently being successfully operated under the scope of the YPACK EU project, in which the selection reactor is supplemented with nitrogen (ammonia) from a total C/N ratio of 100/10.

Keywords: proteins; mixed microbial cultures (MMC); cheese whey; polyhydroxyalkanoates; microbial characterisation; nitrogen source

1. Introduction

Polyhydroxyalkanoates (PHAs) production by mixed microbial cultures (MMCs) from industrial wastes/by-products has been gaining interest as a cost-effective approach for lowering PHA production costs [1]. Nevertheless, this process still requires improvements for achieving lower operational costs. The MMC PHA production from wastes generally comprises three stages, the acidogenic fermentation, culture selection, and the PHA production. The core of the process, though, is the selection stage, since the selected MMC is the central component for determining the efficacy of the PHA-producing process in terms of its storage capacity and productivity.

The culture selection stage is grounded in two efforts, (1) to enrich a MMC in PHA-accumulating organisms capable of producing high amounts of PHA; and (2) sustain a high-growth of the enriched MMCs for the PHA production stage, in order to increase the volumetric productivity of the global MMC PHA production process [1]. Therefore, in the selection stage, both growth and PHA storage need to be supported. Consequently, all nutrients required for growth must be available during this stage. On the other hand, in the PHA production stage, the presence of various nutrients is generally related to lower storage yields and rates, biomass PHA contents, and PHA productivities [2,3]. However, a recent study has shown that PHA productivity increases when nitrogen and phosphorus were present [4].

Many industrial wastes/by-products feedstocks (paper mill wastewater, sugarcane molasses, olive oil mill wastewater, glycerol, oxalate) used so far for PHA production, are rich in carbon compounds but poor in nitrogen and phosphorus, making nutrient supplementation necessary during the culture selection [3,5–8]. Other feedstocks, such as cheese whey, possess potential sources of nitrogen, like proteins, but its availability may be limited by the culture's capacity to metabolise these proteins.

Cheese whey is a by-product of dairy industries. Due to its high protein-content, ca. 1.4–33.5 $g \cdot L^{-1}$ [9], CW may be valorised through the production of whey protein, whose market has been increasing, both for the concentrate, and for individual proteins [10,11]. However, only approximately 50% of the ca. 180–190 $\times$ 10^6 tons cheese whey produced worldwide, yearly, is processed into various food and feed products [12]. The remaining surplus has to be disposed of, representing a severe environmental problem due to its high pollutant characteristics [9], and high volumetric production (9 kg cheese whey is created per 1 kg of cheese produced). In Europe alone, the annual surplus of cheese whey which is not further valorised is 13 $\times$ 10^6 tons, containing about 6 $\times$ 10^5 tons of lactose [13].

The recovery of whey proteins from CW generates a stream containing 81% of the lactose originally present in the milk, which has been demonstrated to be a suitable carbon source for PHA production by pure cultures [14]. Recently, Duque et al. [15] demonstrated that raw CW is a promising feedstock for MMC PHA production, showing that anaerobic fermentation bacteria were able to use a fraction of the protein content present in CW (ca. 30% of the initial protein content in the feed). Due to the presence of an easy fermentable carbon source, sugar (lactose), it is supposed that proteins were consumed as nutrients source, excluding the need for external nutrients supplementation, in the first stage of the process. However, despite the obtained fermented CW still containing a substantial protein content, in that study the aerobic selection stage feed was supplemented with excess nutrients, not assessing for the possibility of the aerobic culture being able to use proteins as nutrients source. Indeed, the ability of anaerobes to hydrolyse proteins is well recognised [16,17]. Hitherto, no study on the impact of proteins in the MMC PHA production has ever been reported.

The use of CW proteins as nitrogen source for selection of a PHA-accumulating MMC was assessed in this work. If an efficient PHA-accumulating MMC capable of using proteins as nutrients source is selected, the operational costs of the process could be reduced, since supplementation of external nutrients sources may be eliminated or decreased. Additionally, the environmental impact of the process effluent would be reduced through removal of the proteins' organic load-fraction. On the other hand, since nutrient limitation has been associated with reaching a higher maximum biomass PHA content [3,18], the ability of the selected MMC to use proteins–nitrogen might disturb the PHA-production stage. Thus, if the proteins–nitrogen is available for the MMC, it may impact the productivity of the process.

This study investigates the possibility of selecting an efficient PHA-producing MMC capable of using the cheese whey proteins as a nutrient source. This would eliminate the need for external nutrients supplementation, thus, reducing the process complexity and costs, while simultaneously producing a less polluted final effluent, thereby, enhancing the waste treatment.

2. Materials and Methods

An aerobic culture selection reactor fed with clarified fermented cheese whey (*fCW*), was first operated with excess nutrients supplementation, similar to the conditions applied in a previous work [15], then the nitrogen supplementation was successively decreased, until no external nitrogen was added. The rationale behind the progressive reduction of the supplemented macronutrient was to select a PHA-producing MMC capable of using cheese whey proteins as nitrogen source.

2.1. Acidogenic Fermentation

The cheese whey (CW) acidogenic fermentation was carried out in an anaerobic membrane bioreactor (AnMBR) comprised of a 10 L glass bioreactor (BioStat®, B-Plus Sartorius), coupled to a hollow-fibre ultra-filtration module (5×10^5 MW cut-off, UFP-500-E-4X2MA, GE Healthcare) and operated under continuous flow. The fCW was obtained as a permeate and biomass was recirculated back to the bioreactor. The AnMBR feeding was obtained by diluting sweet whey powder (supplied by a Portuguese cheese factory—Bel Portugal, Fábrica da Ribeira Grande). It consisted of the following composition (wt.%): lactose 79.3; proteins 9.1; fats 0.7; ashes <8.5, with tap water to achieve a final concentration of 15 g-sugar·L^{-1}. No nutrient (nitrogen and phosphorus) were supplemented, since previous experiments had shown that the CW contains protein and other components which are used as a nutrients source for the anaerobic culture metabolism [15]. The anaerobic bioreactor was operated under constant conditions of mixing, 300 rpm, temperature, 30 °C, and pH, 6 by dosing 2 M NaOH. The organic-loading rate (OLR), hydraulic (HRT), and sludge retention times (SRT) were kept constant (Table 1).

Since the focus of this study was on the enrichment of a PHA-accumulating culture stage, it was necessary to ensure that the fCW fed to the selection reactor was constant throughout the entire operation. Hence, 20 L of fCW were collected under sterile conditions, during ca. 3 days of AnMBR stable operation, fully characterised (Table 1), and preserved at −20 °C, in individual 0.5 L packages, prior to being used as feedstock for the following experiments.

Table 1. Summary of the operational conditions tested and the performance of the AnMBR, and fermented cheese whey characterisation.

Operational Parameters	
OLR	
(Cmol·L^{-1}·d^{-1})	0.53
(gCOD·L^{-1}·d^{-1})	20
Feed proteins	
(gProt·L^{-1})	1.7
Feed Total-N concentration	
(Nmmol·L^{-1})	26.4
HRT	
(d)	1
SRT	
(d)	3
Stable operation time	
(d)	34
Performance Parameters	
$\mathbf{Y_{FP/S}}$	
(Cmol-FP·Cmol-S^{-1})	0.64 (0.05)
(gCOD-FP·gCOD-S^{-1})	0.72 (0.06)
Protein removal	
(gProt%)	30 (7)

Table 1. *Cont.*

Collected fermented cheese whey	
Volume (L)	20
Time of AnMBR operation (d)	3
Substrates concentration (Cmmol-HOrgs·L^{-1})	128 (9)
(gCOD-HOrgs·L^{-1})	4.6 (0.3)
HOrgs [HAc/HBu/HVa/HPr/HLa] (Cmol%)	46(1)/44(1)/5(1)/4(1)/0.3(0.2)
(gCOD%)	41(1)/49(1)/6(1)/4(1)/0.3(0.2)
Ethanol concentration (Cmmol·L^{-1})	2.9 (0.4)
(gCOD·L^{-1})	0.37 (0.05)
Proteins concentration (gProt·L^{-1})	1.3 (0.3)
Total-N concentration (Nmmol·L^{-1})	6
Ammonia-N concentration (Nmmol·L^{-1})	0.78 (0.11)
Total-P (Pmmol·L^{-1})	3.1 (0.8)
Phosphate phosphorus (Pmmol·L^{-1})	0.89
C/N/P ratio (Cmol/Nmol/Pmol)	100/5/3

The listed values are averages and associated standard errors of the mean (SEM), in brackets.

2.2. MMC Enrichment in PHA-Accumulating Organisms

The PHA-accumulating cultures selection was accomplished in a 0.8 L working volume sequencing batch reactor (*SBR*), inoculated with a PHA-accumulating MMC acclimatised to fCW, and operated under similar conditions to those of the reactor, from which the inoculum was harvested [15]. Briefly, the SBR cycle length was 12 h, comprising four discrete phases, influent filling (5 min), aerobiosis (680 min), settling (20 min), and supernatant withdrawal (15 min). The HRT was kept at 1 day, and an OLR of 40 Cmmol L^{-1}·d^{-1} was applied. The reactor was operated in a temperature-controlled room (23–25 °C). The SRT was kept at 4 days by purging 125 mL of mixed liquor at the end of each aeration phase (end of famine). Allylthiourea was always added to the diluting mineral solution (10 mg·L^{-1}) to inhibit nitrification. Air was supplied through a fine bubble diffuser at an aeration rate of 1 vvm (1 L·L^{-1}·min^{-1}), controlled by a mass flow controller (GFC17A, Aalborg). Mixing was kept at 300 rpm during the influent filling and the aerobiosis phases. Proteins present in the fCW were shown to start precipitating at a pH higher than 8.8 (data not shown), thus, whenever the pH reached a value above 8.8, an automatic dosing of 1 M HCl was performed. The pH ranged from 8.0 to 8.8. Pumping (fill and withdraw), aeration, and mixing were automatically controlled by a software program developed within the research group, which also permitted online pH and dissolved oxygen concentration (DO) data acquisition, allowing the online monitoring of the biological process.

In order to assess the possibility of not supplementing nitrogen in the selection reactor, the SBR was initially operated under a "reference operation conditions" (Period I), being supplemented in a similar manner as reported by Duque et al. [15], i.e., a mineral solution of NH_4Cl was added resulting in a supplemented C/N ratio of 100/10 (without taking into account the N present in the fCW, Table 2).

The SBR was stably-operated for sixteen days, under the reference conditions. In the subsequent operational periods, the nutrients supplementation was gradually decreased by reducing the NH_4Cl concentration in the mineral solution. fCW with the organic acids (HOrgs) as carbon substrates, was diluted in a constant volume of a mineral solution, with varying concentrations of nitrogen (NH_4Cl), to impose the different C/N ratios, which were, to a half in Period II; to a quarter in Period III; to an eighth in Period IV; and finally in Period IV the SBR was operated with no external nutrients supplementation. The phosphorus present in the fCW, originated a stable ratio of 100/3 Cmol/Pmol, throughout the SBR operation.

Table 2. Summary of the operational parameters applied to the selection SBR on the five different periods.

Periods	I	II	III	IV	V
Operational Parameters					
OLR ($Cmmol \cdot L^{-1} \cdot d^{-1}$)	42 (4)	45 (1)	44 (4)	43 (1)	38 (4)
fCW C/N ratio (Cmol/Nmol)	100/5	100/5	100/5	100/5	100/5
Supplemented C/N ratio (Cmol/Nmol)	100/10	100/5	100/2.5	100/1.25	100/0
Final C/N ratio (Cmol/Nmol)	100/15	100/10	100/7.5	100/6.25	100/5

The listed values are averages and associated standard errors of the mean (SEM), in brackets.

2.3. MMC PHA-Accumulation Performance

The PHA-accumulation performance of the selected cultures was assessed through fed-batch tests carried out without nutrients supplementation, with continuous aeration and mixing. These tests were performed in a 1 L working volume reactor. When the SBR operation was stable at each period, a sample of the enriched culture was collected, and the fCW was fed, with no nitrogen supplementation, until the cells reached its maximum PHA-accumulating capacity. Air was supplied through a ceramic diffuser and mixing was provided by magnetic stirring (300 rpm). Air flow rate was controlled at 1 $L \cdot min^{-1}$ (1 vvm), by a mass flow controller (GFC17A, Aalborg). For PHA accumulation tests, a biomass purge (0.4 L) was collected from the SBR at the end of the famine phase (ca. 12 h from cycle started). The fCW pH was adjusted to 8 by adding 4 M NaOH. The accumulation tests were then carried out without pH control, at room temperature (23–25 °C). DO was continuously acquired. The PHA accumulation experiments were performed by feeding the fCW pulse-wise, controlled by DO. When DO increased, a new pulse of fCW was injected. This procedure was repeated until no DO response was observed.

2.4. Analytical Procedures

The SBR's operation was followed through the online monitoring of the DO and the pH response, along all cycles, allowing to assess the feast-phase length. Additionally, at least once a week, an entire cycle was closely monitored by collecting samples of the mixed liquor, along the cycle, in order to assess the substrates consumption, biomass growth, and the PHA production. Furthermore, when fast transient periods were expected after a perturbation was induced in the reactor, samples for biomass concentration determination were collected, each for 2–3 days. During the PHA accumulation fed-batch tests, samples were collected periodically during all the pulses in order to assess the substrates consumption and the PHA production.

Biomass concentration, i.e., cells (X) plus PHA, was estimated as volatile suspended solids (VSS) concentration, determined by filtration, according to the standard methods [19].

Organic acids and ethanol were quantified in filtered samples (0.45 μm) through high-performance liquid chromatography (HPLC), using a Merck-Hitachi chromatographer, equipped with an RI detector

and Agilent MetaCarb 87H pre-column and column (column temperature 30 °C, 0.001 M H_2SO_4 eluent, flow rate 0.5 mL·min^{-1}). The HOrgs concentrations were calculated from the standard calibration curves (25–1000 mg·L^{-1} of each organic acid).

PHAs were quantified by gas chromatography (GC) using a method adapted from Serafim et al. [18]. Briefly, a defined amount of lyophilised biomass was incubated for methanolysis, in a 1 mL acidic methanol (20% H_2SO_4) and extracted with chloroform (1 mL). The mixture was digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (2 µL) into a gas chromatograph, equipped with a flame ionization detector (Bruker 430-GC) and a BR-SWax column (60 m, 0.53 mm internal diameter, 1 mm film thickness, Bruker, Billerica, MA, USA), using helium as carrier gas, at 1.0 mL·min^{-1}. The temperature regime started at 40 °C, increased to 100 °C at a rate of 20 °C·min^{-1}, to 175 °C at a rate of 3 °C·min^{-1}, and reached a final temperature of 220 °C at a rate of 20 °C·min^{-1}, ensuring cleaning of the column after each injection. Injector and detector temperatures were 280 °C and 230 °C, respectively. Through the obtained chromatograms, it was confirmed that the PHA in the samples was solely constituted by PHBV polyhydroxybutyrate-hydroxyvalerate (PHBV). 3-HB and 3-HV monomers concentrations were determined through the use of two calibration curves (0.1–10 g·L^{-1}), one for each compound, using standards of a commercial PHBV (88/12 wt.HB/HV%, Sigma, St. Louis, MO, USA), and corrected using heptadecane as an internal standard (concentration of approximately 1 g·L^{-1}).

Protein content in filtered samples (0.45 µm) was determined spectrophotometrically, at 750 nm, by the alkaline copper method, as described by Lowry et al. [20]. Bovine serum albumin (BSA) was used as a protein standard for determining the calibration curve (0–200 mg·L^{-1}).

Ammonia and phosphate concentrations were determined in the filtered samples (0.45 mm) using a segmented continuous flow analyser (Skalar SNA^{++}). Total nitrogen and total phosphorus concentrations were determined using cuvette test kits (Hach®, Loveland, CO, USA).

2.5. Microbial Community Assessment by Fluorescence In Situ Hybridisation (FISH)

Fixation of biomass samples in 4% paraformaldehyde (PFA) and FISH analysis were performed according to Amann [21]. The fluorescently-labelled oligonucleotide probes used were as follows. EUBmix, for all Bacteria (a mixture of probes EUB338 [22]; EUB338-II and EUB338-III [23]; Azo644 for most members of the *Azoarcus* cluster [24]; THAU832 for *Thauera* [25]; PAR651 for *Paracoccus* [26]; and LAMP444 for *Lampropedia* [27]. The four former specific probes were selected based on previous literature [1,28] and the latter was tested based on the result from the detection of *Lampropedia*'s typical morphology. Hybridised samples were viewed with a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM). FISH quantification of Cy3-labelled *Azoarcus*, *Thauera*, *Paracoccus,* and *Lampropedia* in respect to all Bacteria (Cy5-labelled) was done by image analysis, with the Daime software [29], which determines the biovolume fraction of the specifically-labelled target population, relative to the biovolume of the total bacteria. The standard error of the mean (SEM) was calculated as the standard deviation, divided by the square root of the number of images. Nile Blue staining [30] was performed on biomass samples, collected throughout the study, to visually assess the proportion of cells containing intracellular PHA granules.

2.6. Calculations

Feast/famine ratio (F/f, h·h^{-1}) was determined as the ratio between the lengths of the feast phase (F) (the period of exogenous carbon availability) and the famine phase (f) (the period during which there is no exogenous carbon), of the SBR cycles. The PHA content in the biomass was determined in terms of the percentage of VSS on a mass basis, considering VSS to be constituted by cells (X) and the PHA (Equation (1)).

$$\mathrm{PHA(wt.\%)} = 100 \times \frac{\mathrm{PHA(gPHA)}}{\mathrm{VSS(gVSS)}} \quad (1)$$

For expressing X in Cmol (44.2 Cmmol-X·gX^{-1}), the generic chemical formula $C_5H_7NO_2$ was considered [31]. The PHA produced in each cycle (ΔPHA, wt.%) was determined as the maximum PHA content (PHA_{max}, wt.%) during the cycle, generally achieved at the end of the feast phase, minus the PHA content at the end of the famine phase.

Specific HOrgs consumption rates ($-q_{HOrgs}$, Cmol-HOrg·Cmol-X^{-1}·L^{-1}), specific protein consumption rate ($-q_{Prot}$, gProt·gX^{-1}·L^{-1}), and specific PHA storage rate (q_{PHA}, Cmol-PHA·Cmol-X^{-1}·h^{-1}), were determined from the linear regression of the experimental data of each of the HOrg, protein, and the PHA-specific concentrations, respectively, plotted over time. Storage yield (Y_{PHA}, Cmol-PHA·Cmol-$HOrgs^{-1}$) was calculated as the ratio between the q_{PHA} and the $-q_{HOrgs}$. In the accumulation experiments, the specific rates and yields were calculated as described before, for each pulse. In order to compare the different accumulation tests, the average values of each parameter on the first three pulses, were considered.

The standard errors associated with all the determined stoichiometric and kinetic parameters were estimated using standard errors propagation formulae (95% confidence level).

3. Results

3.1. Culture's Dynamic Performance

The objective of this study was to evaluate the potential of selecting a PHA-producing MMC capable of using proteins as a nutrients source, eliminating the need for nutrients supplementation. The fCW had a C/N ratio of 100/5 (Table 2). If all the nitrogen content in the proteins of the fCW fed to the selection reactor was used for growth by the PHA-producing organisms, and assuming the general biomass formula ($C_5H_7NO_2$) and the OLR and SRT applied in this study (40 Cmmol·L^{-1}·d^{-1} and 4 d, respectively), it would sustain a biomass concentration of ca. 0.9 gVSS·L^{-1} in the SBR.

The experiments started with nitrogen supplementation at the levels reported in Duque et al. [15] (Period I) and was gradually decreased until being totally removed, from operational Period I to V. The dynamic system's response to the imposed changes was depicted in the biomass concentration and the F/f ratio trends along time (Figure 1).

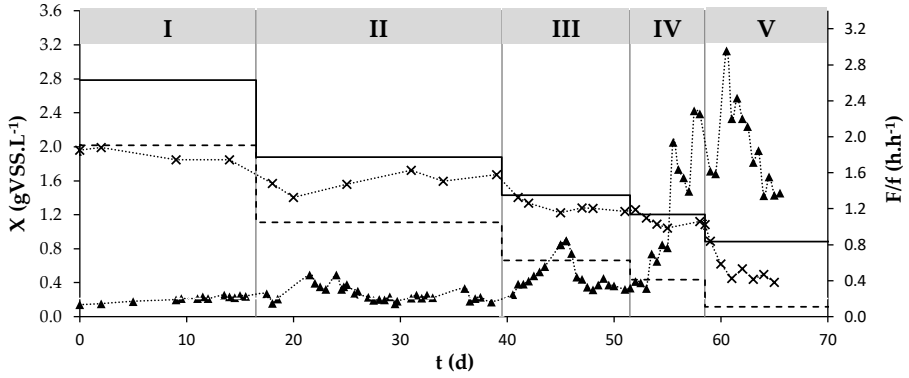


Figure 1. Biomass concentration (*X*, ×) and feast/famine ratio (*F/f*, ▲), over time, in the selection SBR, operated under the five operational periods (I-IV), and representation of the calculated X, if the total nitrogen (*solid line*) and all the ammonia–nitrogen (*dashed line*), present in the feed, were used for cellular growth.

In Period I, cellular growth was chiefly supported by the ammonia–nitrogen, since the observed biomass concentration (average 1.8 gVSS·L^{-1}) was close to that estimated if all ammonia in the feed, both supplemented and present in the fCW, was used for growth (2.0 gVSS·L^{-1}). This reveals that even though the proteins were consumed (in average 27.7 mg per cycle, ΔProt, Table 3), protein–nitrogen was negligibly used by the cells, when a fair amount of ammonia was available. Under the reference operation conditions of Period I, the selected culture was efficient in storing PHA, accumulating ca.

22 wt.%, during the cycles, with a storage yield of 0.5 Cmol-PHA.Cmol-HOrgs^{-1} and a specific storage rate of 0.18 Cmol-PHA.Cmol-X^{-1}·h^{-1} (Table 3).

Table 3. The average performance of the enriched MMC in the selection reactor in the five different periods (I–V); and an overview of the PHA-accumulation assays with the MMC selected on Periods I, II, and III.

Periods	I	II	III	IV	V
Selection Performance Parameters					
ΔPHA (wt.%)	22 (1)	22 (3)	12 (1)	5 (1)	2 (1)
$\mathbf{Y_{PHA}}$ (Cmol-PHA·Cmol-HOrgs^{-1})	0.50 (0.05)	0.55 (0.06)	0.35 (0.04)	0.23 (0.05)	0.06 (0.02)
$\mathbf{q_{PHA}}$ (Cmol-PHA·Cmol-X^{-1}·h^{-1})	0.18 (0.01)	0.17 (0.02)	0.07 (0.01)	0.05 (0.01)	0.01 (0.00)
$\mathbf{-q_{HOrgs}}$ (Cmol-HOrgs·Cmol-X^{-1}·h^{-1})	0.37 (0.03)	0.24 (0.02)	0.20 (0.01)	0.12 (0.02)	0.18 (0.13)
$\mathbf{-q_{Prot}}$ (mgProt·gX^{-1}·h^{-1})	6.2 (1.5)	6.4 (2.1)	11.6 (2.0)	11.6 (2.4)	16.7 (1.2)
ΔProt (mgProt·cycle^{-1})	27.7 (0.2)	32.5 (3.0)	48.5 (2.3)	76.2 (2.0)	79.7 (1.4)
Accumulation Performance Parameters					
ΔPHA (wt.%)	43	31	14	-	-
PHA composition (wt.%HB/wt.%HV)	89/11	92/8	92/8	-	-
$\mathbf{Y_{PHA}}$ (Cmol-PHA·Cmol-HOrgs^{-1})	0.85 (0.12)	0.76 (0.13)	0.52 (0.07)	-	-
$\mathbf{q_{PHA}}$ (Cmol-PHA·Cmol-X^{-1} h^{-1})	0.17 (0.02)	0.18 (0.03)	0.11 (0.00)	-	-
Productivity (gPHA·L^{-1}·h^{-1})	0.20	0.25	0.12	-	-

The listed values are averages (Selection Performance Parameters) or calculated values (Accumulation Performance Parameters) and associated standard errors of the mean (SEM), in brackets. Nr. of measurements: (I:5; II:6; III:6; IV:5; V:7).

When the nitrogen supplementation was diminished to half, from Period I to II, a reduction on biomass concentration was initially observed. Consequently, due to the lower concentration of cells in the reactor, the feast-phase increased, which was reflected in the increase of the F/f ratio (Figure 1). Nevertheless, the system promptly recovered, and after 9 days, during which the culture was probably acclimatising to the new conditions, both the biomass concentration and F/f ratio steadied.

The biomass concentration stabilised in a similar value to that obtained in Period I (1.7 ± 0.1 and 1.8 ± 0.1 gVSS·L^{-1}, Period II and I, respectively), which could not be supported solely by the supplied ammonia consumption (estimated value of 1.1 gVSS·L^{-1}), indicating that proteins were partially used as a nitrogen source during Period II (in average, ca. 32.5 mg consumed per cycle, Table 3). The F/f also stabilised, approximately, in the value attained in Period I (0.20 ± 0.1 and 0.22 ± 0.1 in Period I and II, respectively, corresponding to feast periods of ca. 2 h). The F/f ratios in Periods I and II were within the range of values (F/f of ca. 0.2) which are generally recognised to induce the selection of organisms with a strong storage-response [32].

The efficient selection of a PHA-storing culture in Periods I and II was confirmed by the culture's performance parameters, both in the selection reactor and in the accumulation tests (Table 3). In the selection reactor, the enriched-culture produced a relatively high amount of PHA, during the cycles (ΔPHA = 22 wt.%), indicating that, even though high PHA contents were achieved, during the feast phase, the microbial culture exhausted all the stored reserves, during the famine phase. Consequently, the culture was truly subjected to the selective pressure of feast and famine regime, which enhanced the PHA-accumulating organisms' proliferation.

The specific PHA production rates observed in Periods I and II (0.18 and 0.17 Cmol-PHA·Cmol-X^{-1}·h^{-1}, respectively), were in the range, or higher, than those reported in the literature for similar systems, operated with different fermented feedstocks (0.05–0.11 Cmol-PHA·Cmol-X^{-1}·h^{-1} obtained with fermented molasses [33], and 0.06 Cmol-PHA·Cmol-X^{-1}·h^{-1} with fermented paper mill effluent [3]). However, the obtained values were slightly lower than the maximum values, previously obtained, with fCW by Duque et al. [15], 0.27 Cmol-PHA·Cmol-X^{-1}·h^{-1}. This difference might be related, either to the different OLRs applied (54.4 and 43.0 Cmmol·L^{-1}·d^{-1} in Duque et al. and in the present study, respectively), or to the different organic acids composition. Nevertheless, both cultures selected in Periods I and II showed good and similar storing-capacities during the accumulation tests, reaching storage yields of 0.85 and 0.76, and PHA productivities of 0.20 and 0.25 gPHA·L^{-1}·h^{-1}, respectively (Table 3).

The decrease of nitrogen supplementation to a quarter of the reference (Period II to III), resulted in immediate perturbations in the biomass concentration and, consequently, in the F/f ratio (Figure 1). The biomass concentration decreased after the perturbation, and it did not recover, stabilising in a slightly lower value than that observed in the previous period (1.3 ± 0.1 and 1.7 ± 0.1 gVSS·L^{-1} in Periods III and II, respectively). The achieved biomass concentration was twice that supported by the supplied ammonia (estimated value of 0.6 gVSS·L^{-1}, Figure 1), indicating a larger amount of nitrogen was obtained from proteins (protein consumed per cycle increased to 48.5 mg, Table 3). The F/f ratio increased consistently, up to 0.8, during the initial six days, but it did partially recover eight days after the perturbation was imposed. However, due to the biomass concentration decrease, it stabilised at a higher value than in the previous operational periods (0.35 ± 0.02, corresponding to feast periods of ca. 3 h).

Even though a community capable of using proteins as a source of nutrients was selected, its PHA storage capacity was lower than that of cultures selected in the two initial operational periods. This was probably related to the lower selective pressure which it was subjected to (higher F/f). Indeed, lower amounts of PHA were produced (12 and 14 wt.%, in the selection reactor, and in the accumulation assay, respectively, Table 3), and lower, specific PHA-production rates were attained (0.07 and 0.11 Cmol-PHA·Cmol-X^{-1}·h^{-1} in the selection reactor and in the accumulation assay, respectively, Table 3). Reductions of ca. 30% in the storage yield (0.35 and 0.52 Cmol-PHA·Cmol-$HOrgs^{-1}$ in the selection reactor and in the accumulation assay, respectively, Table 3) and of ca. 50 % in the productivity on the accumulation assay were achieved. Regardless of the change in PHA-accumulating performance of the culture, the monomer composition of the produced PHA was approximately the same, in average 91(1)/9(1) wt.%HB/wt.%HV (Table 3).

When the nutrients supplementation was decreased from one-quarter to one-eighth of the reference, i.e., from Period III to IV, the F/f increased sharply (from 0.35 ± 0.02 in Period III to 1.84 ± 0.15, corresponding to feast periods of ca. 8 h in Period IV). However, this increase was not related to the biomass diminution, as the biomass concentration just slightly decreased (from 1.3 ± 0.1 to 1.0 ± 0.1 gVSS·L^{-1} from Periods III to IV), reaching a value close to that estimated for the condition when total nitrogen supplied to the system was used for cellular growth (1.2 gVSS·L^{-1}). Thus, the selected culture in Period IV was able to successfully retrieve most of the nitrogen present in the proteins. Encouraged by this observation, it was decided to completely remove the nitrogen supplementation, after only 7 days of operating under the conditions of Period IV. This was done, even though, the storage capacity of the selected culture drastically decreased, with low amounts of PHA being produced during the selection cycles (5 wt.%), the storage yield decreased to 0.23 Cmol-PHA·Cmol-$HOrgs^{-1}$, and the specific storage rate decreased to 0.05 Cmol-PHA·Cmol-X^{-1}·h^{-1}, in the selected SBR (Table 3).

Comparing the typical cycles of operation in Period I and Period IV (Figure 2), the loss of PHA-storing capacity of the selected culture becomes clear. In the cycle of Period I (Figure 2a), the culture presented the expected behaviour of a culture enriched in PHA-storing organisms. During the feast phase, most carbon substrates were mainly channelled towards storage (biomass and ammonia

concentrations were almost invariable, during the feast phase, while PHA concentration increased), indicating that the microorganisms were growth-limited. Then, during the famine-phase cells grew at the expense of the stored PHA (during the famine phase a clear decrease of PHA and ammonia, and an increase in biomass concentration were observed). In the cycle of Period IV (Figure 2b), cellular-growth occurred to a significant extent, during the feast phase (from 0.87 to 1.09 gVSS·L^{-1}), possibly indicating that the famine phase was not long enough to induce a selective pressure for PHA-accumulating organisms. The resulting short famine phase, in the consecutive cycles, was thus not enough to discard the non-accumulating organisms from the selected culture. Additionally, the specific protein consumption rate and the total amount of proteins consumed during the SBR cycle in Period I were lower (6.2 mgProt·gX^{-1}·h^{-1} and 27.7 mgProt, Table 3) than in Period IV (11.6 mgProt·gX^{-1}·h^{-1} and 76.2 mgProt respectively, Table 3). In the latter period, proteins were consumed simultaneously to the growth during the feast, indicating that a fraction of proteins was surely used as a nutrients source.

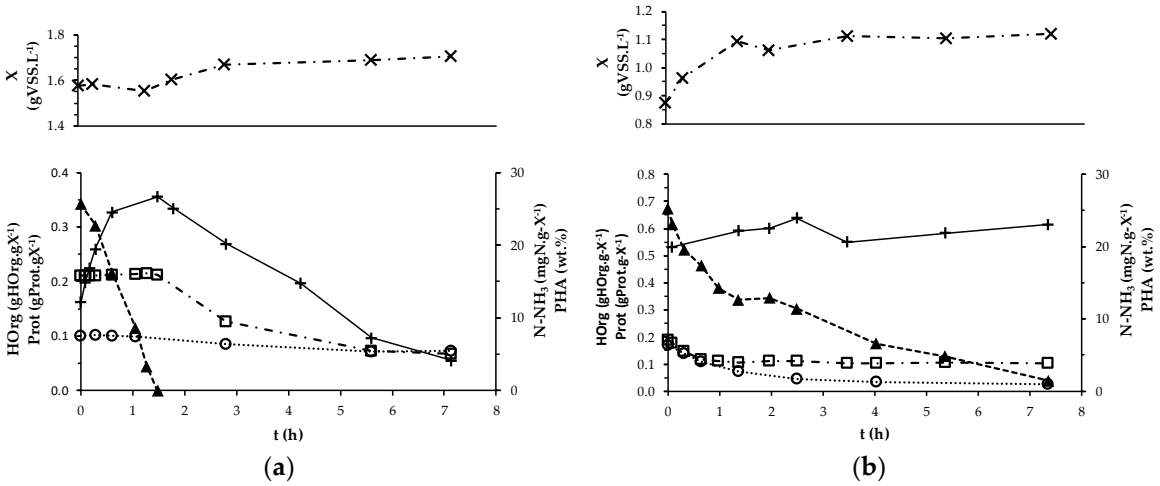


Figure 2. Typical profiles of biomass concentration (*X*, ×), specific concentrations of organic acids (*H.Org*, ▲), proteins (*Prot*, ○) and ammonia–nitrogen (*N-NH_3*, □), and biomass PHA content (*PHA*, +) obtained in SBR's cycles in Period I (**a**) and Period IV (**b**).

When the nutrients supplementation was completely removed, from Period IV to V, the biomass concentration decreased drastically to 0.50 ± 0.03 gVSS·L^{-1}, and consequently, the F/f ratio reached higher values (1.9 ± 0.2, Figure 1, corresponding to feast periods of ca. 8 h). The biomass concentration was still higher than the one that could be supported solely by the ammonia present in the fCW (estimated value of 0.11 gVSS·L^{-1}), indicating that protein–nitrogen was being used. However, albeit the culture selected in Period V was capable of using proteins as the sole nutrients source, only ca. 26% of the protein–nitrogen present in the fCW was consumed. This is likely because of the proteins present in the fCW, which had not been used by the anaerobic culture, were not easily metabolised or could not be completely hydrolysed, due to the short HRT of the selection SBR. Low protein hydrolysis rates have been reported in aerobic cultures [34]. Nevertheless, the selected culture which could use proteins as a nitrogen source was most likely not a PHA-accumulating culture, as storage yield and specific storage rate were close to zero (0.06 and 0.01 Cmol-PHA·Cmol-X^{-1}·h^{-1} respectively, Table 3).

3.2. Microbial Community Assessment

The decrease in the efficiency of selecting a PHA-storing MMC from Period III onwards was also sustained by the quantitative FISH analysis results (Figure 3). The significant decrease in the probe-targeted PHA-storing organisms was observed throughout the study. During Period I, the probe-targeted organisms covered 47% of the bacterial population, comprising mostly members of the *Thauera* and the *Lampropedia* genera. *Thauera, Azoarcus,* and *Paracoccus* were previously described as important PHA-storing organisms in the PHA-producing systems fed with fermented feedstocks [28,33].

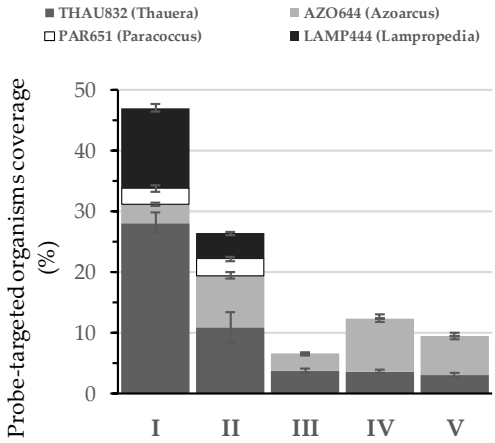
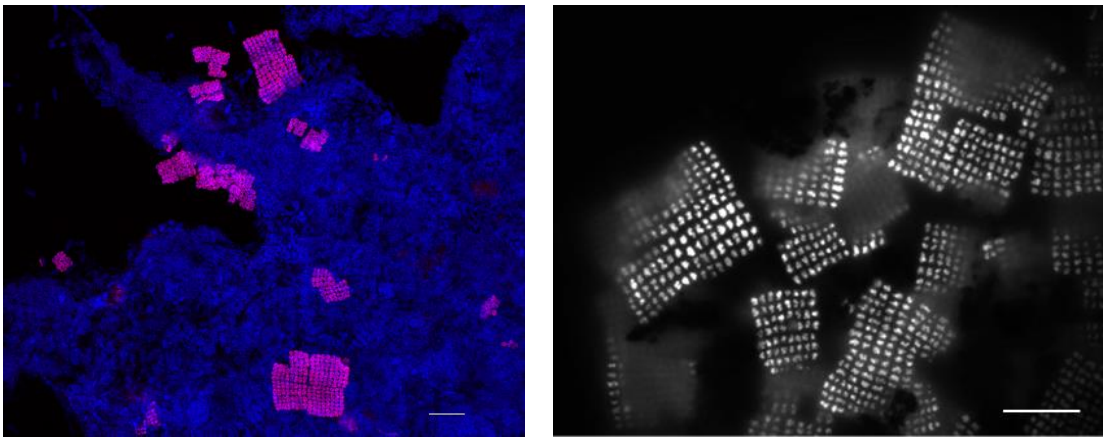


Figure 3. Summary of the microbial population-changes of the enriched MMC, in the selection reactor during the five different periods (I–V).

Lampropedia was found to be capable of storing PHA in an enhanced biological phosphorus removal systems [35], and was identified as an important population in the PHA-production MMC through denaturing gradient gel electrophoresis (DGGE) [36]. Their ability to produce PHA was also confirmed in this study through Nile Blue staining (Figure 4). Members of this genus were previously described in an activated sludge-treating dairy wastewater [35] and cheese factory wastes [37], and were also detected through 16S rRNA gene clone sequencing, in a PHA-producing system, with fermented molasses [28].



(**a**) (**b**)

Figure 4. The typical tablet-shaped clusters of *Lampropedia* observed in this study: (**a**) FISH micrograph with Cy3-labelled *Lampropedia* (*magenta*) and Cy5-labelled Bacteria (*other Bacteria in blue*); and (**b**) Nile Blue staining. *Bar* = 10 μm.

When the supplemented nutrients were reduced to half of the reference (Period II), the abundance of *Thauera* and *Lampropedia* were reduced to about one-third of their original value. However, the PHA production rate (q_{PHA}) and productivity (Table 3) were not significantly affected, suggesting that other populations took over in adaptation to the new conditions. Indeed, the abundance of *Azoarcus* was temporarily increased, suggesting that this genus was more resilient to a decrease in N and P, in the medium. Nevertheless, after Period III, the total number of probe-targeted organisms decreased, which correlated with the sharp decrease in PHA-storing performance, both in the selection and in the accumulation reactors. This may indicate an outgrowth of the PHA-storing organisms by other heterotrophs that are capable of using proteins as the nutrient source, as was hypothesised above. Indeed, the fraction of cells with a positive signal after Nile Blue staining, clearly decreased

between Period I and V (Supplementary Materials Figure S1). Among the assessed PHA-storing organisms, *Thauera* and *Azoarcus* were those that prevailed at the end of the study, suggesting that, among the probe-targeted PHA-producers, these organisms had the highest capacity to survive in a non-supplemented feed.

4. Discussion

The storage capacity of the cultures, evaluated by the PHA storage yield, tended to decrease progressively, as the nutrient supplementation was reduced (Table 3). In fact, when supplementation was reduced by half (from Period I to II), the storage yield had not significantly changed, which seems to indicate the accumulating organisms were not limited in nutrients, therefore they still dominated the microbial community. However, on the following reductions of nutrients supplementation (Periods III and IV), the PHA storage-yield decreased, while the protein consumption increased (ΔProt, Table 3). Indeed, there was an inverse linear correlation between the PHA storage-yield and the specific protein consumption ($-q_{Prot}$, Table 3). An escalation of events might have caused this. The decrease in an easily bioavailable nitrogen increased the reliance on proteins–nitrogen, and the consequent protein uptake. This was a slower process [34] which required more energy, increasing the feast period (decreasing the selective pressure towards PHA accumulation), on one hand, and routing more carbon to be utilised in the catabolism instead of the anabolism, on the other hand, thus, reducing the PHA yield. Another possibility might be that the PHA-accumulating organisms, lacking the required enzymatic mechanisms, were unable to use the proteins as a nitrogen source, thus becoming growth-limited by nitrogen, and consequently, outcompeted by the non-accumulating organisms capable of using the proteins–nitrogen. The reported observations were supported by the relation between the specific PHA storage rate and the specific ammonia consumption (Figure 5), which additionally pointed to a minimum amount of ammonia-availability (ca. 8 Nmol-NH_3·Cmol-X^{-1}), from which the storage rate drastically decreased. Indeed, the decrease in the PHA-storing efficiency when the selection reactor was supplied with lower amounts of nutrients, is in accordance with the findings of Albuquerque et al. [5] who used sugar cane molasses, a nutrients deficient feedstock. In the referred work, it was shown that the insufficient supply of a nitrogen source, in the selection reactor, led to a decrease in the PHA-storage capability of the selected MMC, over time.

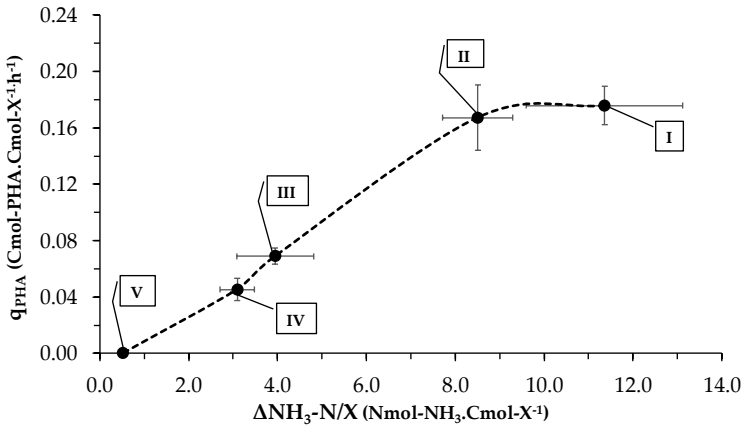


Figure 5. Relation between the specific PHA storage rate (*qPHA*) and the specific ammonia–nitrogen consumption (ΔNH_3-*N*/*X*), during the different periods (I–V).

The loss of selectivity for the PHA-accumulating organisms might also be confirmed by the fractions of ammonia consumed, which were related to the biomass growth, during the feast and famine periods. In a culture efficiently selected on the PHA-accumulating organisms, biomass growth occurred mainly during the famine phase of the FF cycle, at the expense of a stored polymer. Biomass growth, in the SBR cycles, could be indirectly monitored through the ammonia consumption, since nitrification was inhibited through the addition of allylthiourea. The fraction of ammonia used during

the feast increased significantly as nitrogen supplementation was reduced (Period I to Period V) (Figure 6), particularly, after Period III, when the feast phase became longer, suggesting an increase in growth on the external carbon, rather than in storage. These results confirm that the selective pressure for PHA-accumulating organisms was progressively lost from Period II to V. This decrease in the selective pressure could possibly be related to the significant decrease of the biomass concentration (Figure 1).

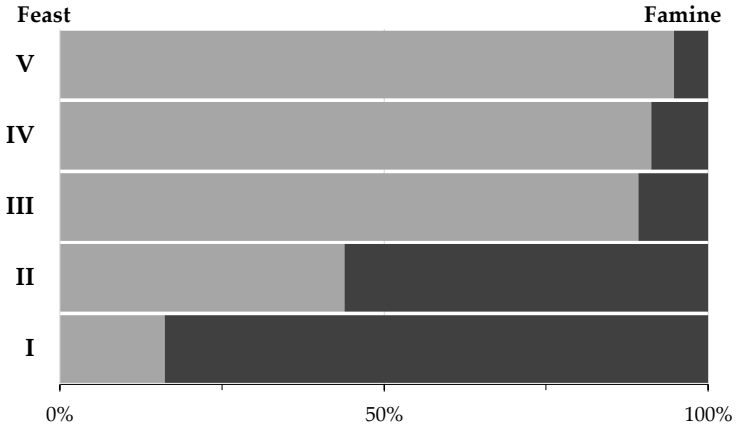


Figure 6. Average fractions of ammonia–nitrogen ($N–NH_3$) consumption in the feast (light grey) and famine (dark grey) phases of the selected SBR, during the five different operational periods (I–V). The consumption percentages are based on the total consumed ammonia–nitrogen, during the entire cycle.

Ultimately, the selection of the PHA-accumulating organisms, under the conditions tested in this study, was strongly affected by limiting the addition of nutrients. Nevertheless, the obtained results suggest that the presence of a proteic nutrients source in the feedstock is not necessarily bioavailable to the PHA-storing organisms, and that an additional process, such as a hydrolysis step between the acidogenic and the selection stages, might be necessary to enable the release of nutrients, from the fCW. Albeit, the economic viability of such alternative measures would have to be assessed.

A 200 L pilot plant (two reactors of 100 L) is currently being successfully operated under the scope of an EU project, YPACK, for the production of PHA from CW by MMC. Based on the results of this study, the selected SBR is being supplemented with nitrogen (ammonia) from a total C/N ratio of 100/10.

5. Conclusions

The objective of this study was to select a PHA-storing culture using cheese whey proteins as the sole nutrients source. Although a progressive selection process was applied to allow a gradual acclimatisation to the limiting ammonia conditions, the selected PHA-producing culture was not achieved. This implies that under the applied conditions, PHA-accumulating organisms could not hydrolyse/assimilate complex nitrogen compounds. The lack of this ability created the proliferation of a flanking-population capable of assimilating protein–nitrogen and overcoming the long periods of famine, with relation to HOrgs, in all probability, by also using protein–carbon.

In conclusion, feedstocks limited in easily bioavailable nitrogen, as cheese whey–proteins, might require additional treatment steps which allow the partial hydrolysis of the nutrients source, or require nutrients supplementation for the efficient selection of PHA-producing cultures. Nevertheless, the cost–benefits analysis of having an additional step for protein hydrolysis or using a cheap external nitrogen source (e.g., urea), as well as the environmental impact of both strategies, need to be evaluated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/8/10/1817/s1, Figure S1: Nile Blue staining (left) and bright field (right) images of biomass samples collected on Period I (a–d) and Period V (e–h) of operation. Stained cells (Nile Blue-positive) indicate the presence of intracellular PHA.

Author Contributions: C.S.S.O. (Conceptualization, Methodology, Validation, Formal Analysis, Writing-Original Draft Preparation, Writing-Review & Editing, Visualization, Supervision); M.O.D.S. (Investigation, Data Curation, Formal Analysis); C.E.S. (Investigation); G.C. (Methodology, Writing-Original Draft Preparation, Writing-Review & Editing); M.A.M.R. (Conceptualization, Resources, Writing-Review & Editing, Project Administration, Funding Acquisition).

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