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The stability of ethanol production from organic waste by a mixed culture depends on inoculum transfer time.

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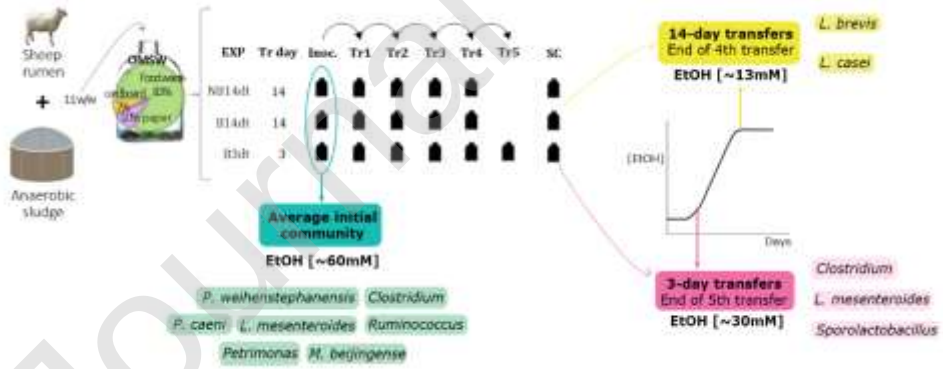
Graphical abstract



Highlights

- Ethanologenic community repeatably enriched from sheep rumen and anaerobic sludge.
- High EtOH production (60 mM) from OMSW at pH ≤ 5.5 in batch microcosms.

- Loss of activity and community composition across sequential inoculum transfers.
- Interval of inoculum transfer critical to maintain ethanologenic activity.
- 3-day transfers stable EtOH activity by enriching *Clostridium* and *Pseudomonas*.

ABSTRACT

Mixed Culture Fermentation is a promising route for bioethanol production from organic wastes. Yet, achieving a stable ethanologenic activity in undefined mixed cultures remains a challenge. This work aimed to retain ethanol production from organic municipal solid waste by microbial communities enriched from sheep rumen and anaerobic sludge mixtures, under low process control (initially aerobic conditions and initial pH ≤ 5.5). To find a stable operating window , sequential inoculum transfer intervals were evaluated (14 and 3-days). Soluble fermentation product profiles and changes in the prokaryotic communities were monitored. The originally inoculated batches always produced high ethanol concentrations (60 mM; 0.070 L_{EtOH}/Kg_{waste}), equivalent to 1/6 of the current corn grain-based ethanol industrial production process. Fermentative activity and community richness significantly decreased in both transfer times regimes tested. However, the 3-day transfer interval led to a stable community which consistently produced ethanol (30 mM) as its main soluble fermentation product. Originally inoculated and 3-day transferred communities consistently enriched for a solventogenic *Clostridium* and an acid-tolerant *Pseudomonas* species. Ethanologenesis, as a dominant catabolic process, is an inherent property of these mixed culture

fermentations, and its maintenance across successive transfers is critically dependant on the inoculum transfer time. [1]

Keywords: Bioethanol, Mixed Culture Fermentation, Organic Waste, Microbial community, Environmental Biotechnology, Waste valorisation

1. Introduction

Bioethanol (EtOH) production from organic waste represents a sustainable route to replace fossil fuels in the transport sector while promoting waste valorisation [1]. Currently, bioethanol industrial and research production processes involve the conversion of feedstocks (mainly crops) into ethanol via defined monoculture or enzymatic fermentation [2]. However, the utilisation of undefined microbial communities, also known as Mixed Culture Fermentation (MCF) [3] is an untapped promising approach for the sustainable production of EtOH, particularly from inherently variable organic wastes, such as the organic fraction of municipal solid waste (OMSW). OMSW is an ever increasing, globally abundant, underutilised resource presently discarded into landfills, dumped into the natural environment, or marginally exploited for heat recovery in industrialised nations [1]. Benefitting from the presence of different microbial populations, MCF has shown potential in the conversion of lignocellulosic feedstocks to EtOH, while having lower requirements for process control [4,5]. In MCF, the enrichment of a microbial community with the desired activity through inoculum sequential transfers is a standard practice, as this selective process [6] is thought to

[1] **Abbreviations**. EtOH: bioethanol/ethanol, OMSW: Organic fraction of Municipal Solid Waste, MCF: Mixed Culture Fermentation

enrich microorganisms that thrive under the selected operating conditions while generating the desired product. In MCF, the success of community selection and its subsequent propagation relies on process design decisions rather than on well-defined features of the organisms under selection [7]**.** Yet, there is no consensus as to when is the most appropriate time to conduct such transfers, with previous studies on EtOH production testing inoculum transfer times ranging from hours to days [4,6,8].

In a previous study by the authors [9], an aerotolerant bacterial community was enriched from sheep rumen fluid and an anaerobic granular sludge (designated as "R+S"). R+S was shown to produce EtOH as its main soluble fermentation product under minimal operating conditions (i.e. static incubations at room temperature, without pH adjustment) in a batch process. This activity was observed when using an unwashed, acid pre-treated OMSW analogue as the only carbon and energy source.

To move toward the development of a stable working ethanol production strategy using the R+S community as a proxy for an aerotolerant ethanologenic MCF, this study aimed to evaluate the functional and structural stability of the R+S community through successive inoculum transfers, under the hypothesis that transfer time would be a determining factor to achieve activity and microbial composition stability. Time-resolved analyses of selected fermentation products and microbial community composition were conducted at two potentially optimal inoculum transfer times including: i) after 14 days, when EtOH production was at its highest, and probably the organisms responsible for this desired activity would be at their most abundant, and ii) an earlier transfer time of 3 days, during the exponential growth phase, which would

potentially result in the selection of a more diverse community able to cope with the initial selective pressures of the medium (i.e., aerobic conditions).

This work proposes and illustrates that tracking fermentative activity and microbial communities' structure and compositions are crucial to determine and mechanistically understand the optimal time for inoculum transfer, thus leading to ethanol production optimisation in mixed culture fermentation [10].

2. Materials and methods

2.1 OMSW medium basic characterisation

To simulate a high organics municipal solid waste typical for overpopulated cities in developing countries, an acid and steam pre-treated OMSW analogue composed of 83% food waste, 10% paper, and 7% packing cardboard, was prepared as previously described in detail [11], and used as the sole substrate in this work. The OMSW analogue's moisture (12.9 % ± 0.4%) and total- (87.1 % ± 0.4%), volatile- (88.8 % ± 0.5%), and fixed solids (9.8 %± 0.4%) contents were assessed in triplicate according to the NREL procedure for moisture content, and the APHA standard method 2540 [12,13]. The OMSW Chemical Oxygen Demand (COD) (19.7 ± 0.3 g/L) and total COD (TCOD) (1.3 gCOD/gOMSW) were determined following previously described methods [11,14,15].

2.2 Microcosms preparation

Microcosms were prepared in 120 mL serum bottles with modified non-reduced medium [8], replacing urea and yeast extract with 2.5g of acid pre-treated OMSW analogue. The bottles were autoclaved for the double purpose of sterilising the culture medium and completing the OMSW analogue diluted acid/steam pre-treatment [16].

After autoclaving, a filter-sterilized trace minerals solution containing $MgCl_26H_2O$ (20 g/L), $CaCl_22H_2O$ (5 g/L), and $FeSO_4{\cdot}7H_2O$ (0.25 g/L) was added to microcosms at a ratio of 1:100 (v/v). The modified non-reduced medium plus the trace minerals solution is hereafter referred to as OMSW medium.

Before inoculation, the pH of the medium was adjusted (Mettler Toledo FiveEasyTM Plus FP20 pH metre, with an InLab pH electrode) for all the originally inoculated and selected successive transfers microcosms to a value of 5.5, an initial pH at which R+S ethanol (EtOH) production had been observed to peak [9]. Adjustment was made with a filter-sterilised 0.1M bicarbonate-carbonate buffer. The original microcosms (before transfer) were inoculated with 1.25g of sheep rumen (from a fistulated wether Suffolk cross breed sheep) and 1.25g anaerobic granular sludge (from a paper waste treatment plant) [9], adding up to 2.5g. The volatile solids content (VSS) for the R+S mixture (tested in triplicate) was $0.05g_{VSS}/g_{OMSW}$. Successive transfers were then inoculated with 2.5g from the corresponding previous culture at selected transfer times. All microcosms were incubated in the dark, at room temperature (~20°C) under static conditions for up to 21 days.

2.3 Experimental design

Three series of experiments were conducted over a 15-month period to test the stability of the R+S community ethanologenic activity by means of successive inoculum transfers (Tr) after its original inoculation (OI) from the source environments: 1) NB14dt: Non-Buffered OMSW medium, with 4 times sequential transfer of the communities enriched after 14 days. 2) B14dt: Identical to NB14dt but with the OMSW medium amended with bicarbonate buffer to adjust all microcosms initial pH to 5.5. 3) B3dt:

Same as B14dt, but with inoculum transfers after 3 days of incubation. Modifications showing improvement in ethanologenic performance were maintained in subsequent systems (e.g. B14dt->B3dt) and are further explained in the relevant results sections. Substrate only Control (SC) microcosms with OMSW media without added inoculum, were included in all sets of microcosms.

All batch configurations were tested in triplicate. Liquid samples were periodically taken from all microcosms, split and either: i) immediately used for pH measurement, ii) further processed for ethanol quantification by Gas Chromatography-FID (GC-FID) or, iii) stored at -20°C for Volatile Fatty Acids (VFAs) Ion Chromatography (IC) and microbial community analyses.

2.4 Analytical methods

GC-FID quantification of ethanol. EtOH concentration was measured using GC-FID, as this method was found to be repeatable and linear for alcohols quantification [11]. For quality control, freshly prepared standard solutions of EtOH were quantified at the start and end of each GC-FID analysis run. 4-methylpentan-2-one (0.8mM) puris. p.a., ACS reagent, ≥99.5% (GC) (Sigma-Aldrich, UK) was used as an internal standard and employed to calculate EtOH concentration in the samples [17].

VFAs IC. On the day of analysis, samples were added (1:1v/v) to 0.1M orthosilicic acid, placed in an ultrasonic bath at 30°C and sonicated for 40 minutes for carbonate removal through acidification and CO_2 effervescence. Samples were then submitted to the Environmental Engineering Lab at Newcastle University for VFAs IC analysis.

Electron balance estimations. Electron balances were conducted to broadly evaluate the recovery of electrons from the OMSW medium (electron donor) in the form of

soluble fermentation products (electron acceptors). The proportion of electrons from the substrate converted into ethanol were calculated as previously described [11]. The OMSW medium had an original input of 4687.5 e-meq./L based on the Biomethane Methane Potential estimate derived COD.

For the electron balances, mmol concentrations of end-products were converted to electron milliequivalents (e−meq.) using electron equivalents per mol values, obtained using the half-reaction method (Rittmann and McCarty 2001). The recovery of e−meq. to total soluble end-products (quantified in terms of mmol of carbon equivalents) is reported as a percentage of the initial total e−meq. provided as substrate.

2.4.1 Statistical analysis of physicochemical data.

RStudio (RStudio Inc., US) with R version 4.0.0 (The R foundation, US) were used to compute 2-way-ANOVA followed by Tukey HSD tests to evaluate whether ethanol maximal production across transfers as well as maximal total productivities in terms of carbon milliequivalents were statistically different. Confidence intervals and error bars throughout this work represent the Standard Error of the mean (SE).

2.5 Microbial community analysis

Genomic DNA was isolated from ~0.5mg of samples from selected microcosms. Due to logistic constraints, DNA was extracted from different samples using one of two different extraction kits i.e. the FastDNA® SPIN Kit for Soil1 (MP Biomedicals LLC, UK) for NB14dt and B14dt and DNeasy PowerSoil Kit2 (Qiagen, UK) for the B3dt microcosm sets. The manufacturer's protocols were followed, accordingly.

2.5.1 Next generation sequencing

Two different NGS platforms were used for microbial community analyses, namely by Ion Torrent PGM sequencing and, MiSeq synthesis sequencing (Illumina, UK). **Ion Torrent PGM sequencing pooled library preparation.** DNA extracts from the NB14dt and B14dt microcosm sets, which included replicates of OI, Tr1 and Tr4 microcosm successions were subjected to PCR amplification of the V4-V5 region of the 16SrRNA gene using the universal primers F515 and R926 [18] with individual Golay barcodes attached to the F515 forward primer. The amplified libraries were purified using an Agencourt Ampure XP purification Kit (Beckman Coulter Inc., UK), following the manufacturer's protocol. An equimolar pool comprising the purified amplicon libraries and two procedural blanks were prepared after each amplicon was quantified using a benchtop fluorometer Qubit Fluorometer 2.0 in combination with a Qubit® dsDNA HS Assay Kit (Life Technologies, UK), following the manufacturer's protocol. The pooled library was sequenced with the Ion Personal Genome Machine® (PGM™) System using an Ion 316™ Chip v2 (Life Technologies, UK) at the Environmental Engineering research laboratory, Newcastle University.

Preparation of samples for MiSeq sequencing. DNA extracts from the B3dt replicates for the OI and Trs 1, 3 and 5 successions plus a procedural blank were submitted to the NU-OMICS research laboratory, Northumbria University, where a 16S rRNA gene V4 region primer set [19] was used for PCR amplification and the library was prepared and validated with the aid of a 2100 Bioanalyzer (Agilent Genomics, UK). Sequencing following a 2 x 250 bp format with V2 chemistry was performed.

2.5.2 16S rRNA sequence library processing and community composition analyses

Raw sequencing data (FastQ files) were processed using Mothur v.1.39.5 [20] following Mothur's 454 and Miseq Standard Operating Procedures for processing Ion Torrent and Miseq sequencing data, accordingly [20,21]. These processes included reads quality trimming (minimum length of 275nt), filtering, alignment, chimera removal via the Uchime algorithm [22], classification, and clustering into operational taxonomic units (OTUs). OTUs were assembled using the OptiClust algorithm [23], producing a distance matrix where any pairwise distance larger than 0.15 was eliminated. Quality sequences were aligned against the SILVA database [24]. OTUs (selected at 97% similarity) identified and their abundances were used to compute □-diversity metrics, namely, richness (S), diversity (Simpson's inverse diversity, D), and evenness (Simpson's evenness, E) indices. OTUs abundances rarefied to the smallest library count in the corresponding data set, were imported into PRIMER 7 (Primer-E Ltd., Plymouth, UK) to compute the Bray Curtis similarity matrix [25]. The Bray-Curtis similarity coefficients were used to produce similarity percentages analyses (SIMPER) to identify both, the most abundant OTUs integrating each sample, as well as the discriminatory OTUs between the sample groups, cumulatively contributing to 70% of the total similarity or dissimilarity between groups [25]. Also, under the assumption that the most abundant OTUs are the most active within a community (Clarke and Gorley, 2015), the transformed data set was filtered to only consider OTUs accounting for >1% of the total number of individuals in each sample. Additional to the taxonomy assignments (up to genus level) for the OTUs made by Mothur (using the SILVA database release 128), the most abundant representative sequence within each OTU was aligned against the 16S

rRNA sequences (Bacteria and Archaea) GenBank database using the megablast algorithm [26].

Phylogenetic and molecular evolutionary analyses were conducted using MEGA X [27]. Evolutionary histories were inferred using the Neighbour-Joining method [28]. Evolutionary distances were computed using the Maximum Composite Likelihood method [29] and the percentage of replicate trees in which the associated taxa clustered together was determined by bootstrap analysis of 1000 replicates [30].

3. Results and Discussion

3.1 Effect of bicarbonate buffer on ethanol production by the R+S community.

In agreement with previously described preliminary tests [9], after 14 days of incubation the OI NB14dt microcosms reached their peak EtOH production (Table 1), with EtOH being the major fermentation soluble product generated (Fig. 1A). Remarkably, this ethanologenic performance was almost double that of preliminary tests (~30 mM, [9]). Regardless, this initially high ethanologenic activity decreased dramatically across transfers (Fig. 1A), dropping successively to 30.02% in Tr1 and 18.71% in Tr4 of the EtOH production observed in the initial inoculation. Importantly, the maximal total productivity in terms of carbon and electron milliequivalents (Table 1) decreased in the same fashion as EtOH production across transfers, losing up to 75.49% and 78.85% of total productivity respectively. Nevertheless, the proportion of electrons used to produce EtOH did not vary drastically (average 59.72 ± 0.93%) suggesting a general loss of metabolic activity as opposed to a change in fermentative pathways.

Despite the initial pH of all the microcosms being adjusted to 5.5 before inoculation, the inoculation of transfers' microcosms (OI-->Tr1…) caused a significant pH drop (p-

values <0.007; 2-way-ANOVA, Tukey HDS tests) of about 0.5 units (Fig 1. A-pH). This difference was attributed to the inoculum type used in each case. Direct inoculation with sludge and rumen, both with close to neutral pH values, did not alter the OI culture medium pH, while successive transfers were inoculated with growth medium with a pH of about 4.5 (Fig. 1A-pH). The substrate only controls maintained a pH close to 5.5 across all transfers, registering a maximal productivity in terms of soluble carbon milliequivalents of about 1mM (0.11% of EtOH produced by R+S communities at Tr4) in all stages of this experiment.

Based on these and preliminary results [9], it was hypothesised that the general loss of fermentative activity was caused by the significantly lower initial pH of the transfers and that further pH adjustments after inoculation would improve the production of soluble fermentation products. This hypothesis was subsequently tested in experiment B14dt (Fig. 1B), where transfer medium initial pH was adjusted to 5.5 after inoculation. **[FIGURE1]**

EtOH production was also dominant in all the B14dt incubations (Fig. 1B), however a similar pattern of fermentative activity loss was observed across transfers (Fig. 1B). Importantly, the maximum EtOH concentration (62.96±7.77) reached in the OI replicates was not significantly different to that observed in NB14td (2-way ANOVA p-value 0.403) nor between corresponding transfers from the two experiments (2-way ANOVA p-value <0.001, Tukey HDS test p-values >0.9). The maximal total productivity in terms of carbon and electron milliequivalents (Table 1) decreased by up to 60.03% and 65.25% of total productivity, respectively, showing only a slight improvement from previous NB14td results. The same statistical analysis was conducted to compare maximal total

productivities in terms of carbon milliequivalents between both experimental systems (2-way-ANOVA p-value 0.128, Tukey HDS test p-values >0.49), also revealing no significant difference in the maximal total fermentative productivity obtained in NB14dt and B14dt. Similar to the NB14dt results, the proportion of electrons used to produce EtOH (average 54.78± 1.25%), suggested a general loss of fermentative metabolic activity as opposed to a change in fermentative pathways.

A 2-way-ANOVA (p-value 0.06) confirmed, albeit with borderline significance, that the initial pH values across B14dt transfers were not significantly different (Fig 1B-pH). Based on these results the hypothesis of initial pH differences being the main factor causing the loss of ethanologenic and fermentative activity was rejected.

3.2 Effect of transfer interval in ethanol production by the R+S community

The B3dt transfer regime was conducted to assess if an inoculum transfer time during the EtOH production exponential phase (Fig. 1) would allow the maintenance of the OI high EtOH production. We hypothesised an earlier transfer time would retain important non-ethanologenic but oxygen consuming community members thus facilitating the onset of anaerobic conditions, and ultimately enabling high EtOH production.

Accordingly, successive transfers 3 days after inoculation were tested. The soluble fermentative activity of the R+S community was monitored for 21 days during the OI incubations, and for 14-day periods for the subsequent transfers.

Consistent with the previous experiments, EtOH was the main soluble fermentation product with a maximal concentration of 56.85 mM ± 2.6 mM reached in the OI microcosms after 14 days of incubation, followed by a slight decrease to 50.96mM ± 8.12 mM at 21 days (Table 1). Although EtOH maximal productivity decreased to an

average of 30.5mM±1.3mM in the subsequent transfers (about 56.2% of the initial production), EtOH remained the major soluble fermentation product and a more stable soluble fermentative activity was achieved by conducting a 3-day transfer regime (Fig. 1C). Another distinction of the 3-day versus the 14-day transfer results, was the relatively stable and high production of butyric acid as the second major soluble fermentation product (Fig 1). Additionally, formic acid, which was not detected in previous experiments, was the third major fermentation product and stably produced throughout the B3dt transfers (Table 1). The pH profiles from the different transfers varied in trajectory, apart from the OI microcosms, however all transitioned from about 5.5 to pH 4.7 at the end of their incubation periods (Fig. 1C).

The maximal total productivity in terms of carbon and electron milliequivalents (Table 1) slightly decreased by the end of the 5th transfer to 81.5% and 78.41%, from the original, respectively, showing a clear improvement in terms of total productivity relative to the 14-days transfer experiments. Similarly, the proportion of electrons used to produce EtOH did not vary drastically across transfers (52.83 ± 1.01%). The electron estimates signify that 13.53% of electrons were recovered in the OI incubations [11], and 14.55% of the electrons fed into the system ended up as EtOH. While by Tr5, 7.36% of electrons from the OMSW were recovered as EtOH.

Statistical analysis demonstrated that although all transfers produced significantly different maximal EtOH concentrations compared to the OI microcosms (2-way-ANOVA p-value 0.00183, significance level 0.001, Tukey HDS p-values <0.008), their production did not differ among themselves (p-values >0.8). From this it can be concluded that a stable ethanologenic activity was achieved.

Despite B3dt's promising results, the significant loss of EtOH production between the OI microcosms and Tr1 observed in all experiments remained a challenge. As the measured physicochemical variables tested did not provide an obvious mechanistic explanation of the results, we analysed the microbial communities' compositional changes in all experiments, evaluating their stability, and inferring which organisms were the key players for EtOH production.

3.3 Community composition dynamics across sequential inoculum transfers

The OI microcosms for all experiments had initially rich and diverse communities (Fig. 2A), as could be expected by their direct inoculation with the combination of sheep rumen and anaerobic granular sludge. However, in both the NB14dt and B14dt systems, a sharp loss of richness and diversity was observed over time to such an extent that effectively 2 OTUs (*Lactobacilli* species) represented more than 90% of the average cumulative community composition by the end of both experiments (Fig. 2B). Although the decrease in richness and diversity could suggest the enrichment of organisms fitted to grow under the specific initial conditions and utilise OMSW as substrate, these results along with those of loss of fermentative activity (Fig.1 A,B) indicate the absence of growth, particularly after transfer from the initially more active OI microcosms in both experiments. To broadly evaluate whether the loss of richness and diversity was mostly due to the absence of growth rather than the dramatic enrichment of particular taxa, the quantities of 16sRNA amplicons for each time point across transfers in both experiments were compared. Post-incubation DNA average concentrations (ng/mL) in NB14dt Tr4 were found to be about 10% of that from the NB14dt OI microcosms. Nevertheless, DNA average concentrations were overall higher in B14dt (~60%) possibly

indicating a positive effect on growth by the initial pH adjustment performed post-inoculation in these systems.

The B3dt OI microcosms had notably richer and more diverse communities at day 0, but also throughout the duration of their incubation period (Fig 2. A). Although the richness of these communities was higher than for that of the 14-days transfers, the diversity levels after inoculation from the OI microcosms remained similarly low in all successive transfers, suggesting the community composition had converged, as before, on a small number of OTUs. However, in this instance the decrease in richness and diversity along with the B3dt fermentative activity results (Fig. 1C) indicated the enrichment of OTUs fitted to grow under the specific conditions and which produced EtOH from the utilization of OMSW as substrate. The fermentation profiles also suggest that the 3 days regime, allowed the retention of ethanologenic and fermentative microorganisms when they are actively growing (exponential phase) and survived the transfer process better than organisms that had entered a stationary phase or which grow later in the succession which occurred with longer transfer times. The contrasting communities enriched by the two inoculum transfer regimes tested and the different selection pressures determining their structure are further discussed below. **[FIGURE 2]**

3.3.1 Specific microbial taxa typical of the different transfers regimes

The three systems' initial communities (OI, day 0) were, in different proportions, highly abundant in members from the genera *Petrimonas, Pseudomonas, Leptolinea, Methanobacterium,* and a *Ruminococcus*-like OTU. From these, *Petrimonas, Leptolinea, Pseudomonas caeni* (≥99% similarity), and *Methanobacterium beijigense* (100% similarity) have all been reported as members of anaerobic sludge [31–33]. After 7 days

of incubation, these taxa remained relatively stable, and all systems highly enriched for members of the genera *Clostridium* and *Leuconostoc.* However, clear differences in composition occurred between the two inoculum transfer time regimes.

Leuconostoc mesenteroides (100% similarity) contributed to at least 30% to the total composition (SIMPER analysis) of both 14-day transfers communities, in agreement with previous observations [9]. The other OTU abundantly enriched (>7% total composition, SIMPER analysis) after 7 days in both communities was *Clostridium saccharoperbutylacetonicum* (≥99% similarity), a solvent-producing clostridial species [34]. Additionally, the NB14dt community was also enriched for other solventogenic clostridia (i.e. *Clostridium beijerinckii* and *Clostridium tyrobutyricum* (99% and 100% similarity, respectively) and *Lactobacillus casei* (99% similarity), a species commonly found in intestinal tracts of animals with optimal growth pH of 5.5 [35], during the OI incubations (Fig. 2B). Apart from these newly enriched OTUs, both communities were still mostly comprised of their 7-day members by the end of the incubation period, with *L. mesenteroides* dominating in both experiments

The initial transferred communities (Tr1, t0) were also dominated by *L. mesenteroides* (>30% contribution). However, the first transfer of NB14dt contained an important proportion of the solventogenic clostridia, with three OTUs cumulatively contributing 21.96% of sequences to the total community composition. In addition, two *Lactobacillus* species contributed about 11% to this initial NB14dt Tr1 composition. The B14dt Tr1 initial community was mostly comprised by the OTUs present in the 14-days community it came from, with *C. saccharoperbutylacetonicum* contributing 11.18% and *Lactobacillus casei* 3.22% of the total community composition, respectively. However,

from day 7 after transfer, *Lactobacillus* species, mainly *L. brevis* (99% similarity) and *L. casei* dominated the enriched communities until the end of both experiments (>90% of total community, SIMPER analysis) (Fig.2B).

Consistent with the fermentation profiles, and the development of acidic pH conditions, the dominant species (*Lactobacillus*) at the end of both the NB14dt and B14dt experiments are known to require an acidic pH for growth [35,36]. The maximal EtOH concentrations generated by the OI microcosms in both 14-day transfers experiments were reached at 14 days of incubation (Fig. 1A,B) suggesting the presence of ethanologenic and ethanol-tolerant bacteria. However, the bulk of the ethanol was produced before 7 days of incubation, a time point at which both communities were mostly comprised by different proportions of *L. mesenteroides, C.saccharoperbutylacetonicum* and *P. caeni*, from which only *L. mesenteroides*, and *C. saccharoperbutylacetonicum* were transferred into fresh media during the next transfer at 14 days. Given that ethanol was still increasing prior to the first transfer, it would be safe to assume that the ethanologenic bacteria were still viable in these microcosms, with *C. saccharoperbutylacetonicum* being likely responsible for subsequent albeit limited EtOH production. This observation of viability therefore indicates problems in the transfer time regime itself. Although they do not participate directly in EtOH production, the role of aerobic bacteria such as *P. caeni* could be crucial for the establishment of the right environmental conditions for fermentative microorganisms to thrive [9]. Despite *Lactobacillus* being facultative anaerobes, their activity does not seem to be enough to provide such conditions.

In the B3dt system, *P. caeni* had the highest individual contribution (6.65%) to the initial OI community composition. After 3 days, although most of the original OTUs remained as part of the community, their contribution to the total composition decreased, while a newly enriched *Pseudomonas* species, 100% similar to two species (*Pseudomonas weihenstephanensis and Pseudomonas helleri*) isolated from raw cow's milk [37], a solventogenic *Clostridium* species (100% similar to *C. beijerinckii, C. saccharoperbutylacetonicum, C. beijerinckii, C. diolis*), and *L. mesenteroides*, contributed with 16.15%, 5.25% and 2.66% to the summative composition (SIMPER analysis), respectively. For simplicity, the pseudomonads and clostridial species will be thereafter referred to as *P. weihenstephanensis* and *C. beijerinckii* as these were at the top of the match list in the GenBank searches.

Since the EtOH concentration at day 3 of the initial incubation (25.32 mM ± 1.06 mM) was almost equivalent to the total maximal concentration reached in the subsequent transfers, the community enriched at this time point played an important role in the total ethanologenic activity observed in these microcosms, which based on the SIMPER results, was only 38.87% dissimilar to that observed on day 0, with the enrichment of the three OTUs mentioned before adding the most to the average dissimilarity between these two communities. The maximal EtOH production was reached at 14 days, a time point at which *C. beijerinckii* was the most abundant OTU (14.44%), remaining dominant to the end of the incubation period. Inoculum transfers were then conducted from the B3dt communities into fresh culture media, with the adjustment of pH to 5.5 after inoculation. Despite the inoculation of the first transfer with a relatively rich community (Fig. 2), after 3 days of incubation, *P. weihenstephanensis* represented more than 50%

of the total community composition, followed by *C. beijerinckii* with an average contribution of 23.63%, thus reaching the 70% similarity cut-off selected for SIMPER analysis. By the end of the incubation period, *C. beijerinckii* was the most abundant OTU, while *L. mesenteroides* was also significantly enriched and the *P. weihenstephanensis* contribution had diminished. The third transfer was mostly dominated by *C. beijerinckii, L. mesenteroides* and a member of the genus *Escherichia*, despite *P. weihenstephanensis* being the dominant OTU at the time of inoculation. By the end of the experiment, *C. beijerinckii, L. mesenteroides, Sporolactobacillus putidus* a spore-forming, microaerophilic lactic acid bacterium with optimum growth at pH 4.5 [38] and an *Enterococcus* genus member each contributed in different proportions to the total community composition in Tr5.

The retention of ethanologenic activity in B3dt (Fig.1 C) is consistent with the continued presence and enrichment of *C. beijerinckii*, a solventogenic bacteria, as opposed to the *Lactobacillus* species dominating the 14-days transfers, thus providing an obvious candidate for the organism principally generating EtOH from the OMSW. Equally, the relatively stable production of butyric acid in the B3dt system can be linked to the continuous enrichment of solventogenic clostridial species, known to produce this volatile fatty acid during the exponential phase in their Acetone-Butanol-Ethanol (ABE) fermentation [39]. Additionally, the distinctive production of formic acid observed in this experiment, matches with the enrichment of *Escherichia* (Fig. 2 B), an enteric gamma-Proteobacteria genus known to conduct Mixed Acid Fermentation, of which formic acid is a product, under anaerobic conditions [40]. These featuresfurther illustrates the effect that inoculum transfer time has on the fermentation products obtained.

3.4 Average ethanologenic initial community enriched in the three experiments.

Despite the differences observed across transfer regimes, the batch of microcosms directly inoculated with the combination of rumen and sludge produced about 60mM of EtOH by 14 days in all experiments, and reached an average total productivity of soluble fermentation products in terms of carbon and electron milliequivalents of about 200mM and 1085.18 e^- meq. /L, respectively (Table 1). Hypothetically recovering about 23.15% of electrons from the OMSW, (based on BMP estimates), from which about 15% of the electrons initially provided by the substrate were used for EtOH production. These results were observed regardless of the time which had elapsed in-between these independent experiments. This consistency lead to the conclusion that the ethanologenic activity after initial inoculation with the combination of these inocula sources under the environmental conditions tested is a reproducible and repeatable process. Ethanologenesis and its dominance as a catabolic process are then an inherent property of these experiments. Clear similarities were also found in the microbial compositions of their initial communities.

Under the assumption that abundance reflects activity, it is deduced that the communities enriched after 7 days of incubation in all the OI batches comprised all the functional capabilities required to achieve such ethanologenic activity. The phylogenetic tree (Fig. 3) comprising the most abundant OTUs enriched after 7 days of incubation in the three OI batches along with close relatives selected from searches (BLAST) of the Genbank database, demonstrates that highly similar organisms where indeed enriched in each independent experiment. The tree further contains information about the

source environments from which close relatives were obtained, with results being consistent with anaerobic sludge and reactors, rumen, and fermented food. **[FIGURE 3]**

The putative functions of some of these OTUs have already been partially discussed [9], with a proposal that the initial high abundance of *Pseudomonas* might be responsible for the depletion of oxygen in both the headspace and liquid media, creating the necessary conditions for strict anaerobes, such as *Clostridium*, to grow [41]. Additionally, biofilms may have been generated by the *Pseudomonas*, creating microenvironments where the community members could perform their metabolic activities while protected from low pH and other growth inhibitors. Potentially, non or poor biofilm formers (e.g. lactic acid producers) as well as the *Pseudomonad* species benefited from environmental protection and nutrients exchange [42,43]. Under this hypothesis, the *Ruminococcus'* likely cellulolytic activity would have benefited the carbohydrate oxidisers (i.e. *P. caeni*) and fermenters (i.e., *Clostridium* and *Petrimonas*), while actively contributing to EtOH production. Nevertheless, it is also possible that the cellulosic fraction of the OMSW was not consumed, as many of the OTUs identified were likely able to grow on fatty acids, peptides, and simpler carbohydrates likely to be in solution due to the *in-situ* hydrolytic substrate pre-treatment. Under the assumption of these inferred functions, the dramatic loss of ethanologenic activity in the 14-day transfers tests could be mostly attributed to the absence of *Pseudomonas* and Clostridial species, where *Lactobacillus casei* and *Lactobacillus brevis* dominated in different proportions in the transferred communities.

More studies would be required to support such a hypothesis; however, a thin biofilm-like layer was observed to develop on the surface of the substrate in inoculated microcosms which was clearly different to the state of the substrate only controls.

3.5 Known and unknown selective pressures influencing the microbial communities enriched.

As hypothesised, inoculum transfer time strongly directed fermentation towards EtOH production, however, other factors likely exerted selective pressure in these systems. For instance, the initial presence of oxygen in the medium at the beginning of each transfer could have imposed a two-fold limitation on the growth of anaerobic bacteria (i.e. *Clostridium*). These two limitations being: i) the anaerobes inability to grow under initially aerobic conditions and, ii) a reduction in the diversity of aerobic organisms originally present in the inoculum across successive transfers. The latter factor which would result in a longer oxygen depletion time, potentially favouring the growth of facultative organisms [44] may have played an important role during the 14-day transfers regime. Here the aerotolerant, acidophilic Lactobacilli which are specialised [45] in degrading decaying-plant material (present in high proportions in the OMSW) , dominated transfer enrichments further limiting the growth of slow-growing competitors by further decreasing the medium pH through fermentative production of fatty acids from an inaccessible substrate for other taxa. Additional to oxygen depletion, acid inhibition caused by the initially low pH of the medium might have been responsible for the loss of richness by the end of the OI incubations, and for the dominance of the acidophilic Lactobacilli in the 14-day regime experiments. These two conditions could have caused further selective pressures, for instance, the dominant growth of a

relatively small group of OTUs enriched in the original inoculations, probably outperforming other organisms in terms of readily available nutrient uptake [46].

The presence of lignin-derived compounds, which the enrichment of bacteria able to degrade aromatic compounds supports (e.g. *Pseudomonas*), is likely to have negatively affected the growth of other inoculum members, including potential ethanologenic bacteria [47]. Another factor that was not optimised for the present study and which probably influenced the results observed is the inoculum to substrate ratio (I/S) in terms of its volatile solids content. The I/S of OI incubations was $0.064 g_{VSR+S}/g_{VSOMSW}$, a rather low value as determined by a relevant study [48] showing that optimal I/S values from an OMSW analogue were reached in the range of 0.12 - $2 g_{VSinoculum}/g_{VSwaste}$. However, optimal I/S ratios not only depend on the inoculum and substrate concentrations, but also vary in relation to the desired end-product.

The present study demonstrates that successive inoculum transfer is not a straightforward process as commonly depicted by MCF batch studies, where the determination of the community composition is done once a stable degradation/fermentation activity is reached for the potential isolation of the most abundant members, or for the general characterisation of the system, without tracking the changes in community composition that occurred to get there [4,8,49], neglecting to explain the rationale behind the selection for the transfer times chosen. In consequence, the literature fails to provide guidance for future studies. The efficiency and cost of ethanol production by MCF could be further optimised by simple interventions such as the evidence-based selection of the inoculum transfer time.

Crop based EtOH production process yields and processing times currently outperform the parameters reported here for OMSW [50], suggesting that there is room for further improvement. Furthermore, R+S based EtOH production as part of a biorefinery project could lead to the development of additional areas of research, as the recovery of other valuable products (e.g. chemicals and biomass) would add value and make the entire process cost effective and firmly embed it in a circular economy philosophy.

4. Conclusions

Batches of microcosms directly inoculated with mixed cultures of rumen and anaerobic sludge (R+S) under initially aerobic conditions and pH≤ 5.5 produced EtOH (60mM; 0.070L_{EtOH}/Kg_{waste}) from OMSW as the main fermentation product in three separate experiments conducted over a 15-month period. A sequential inoculum transfer time at the time of maximal EtOH production (14 days after inoculation) lead to the loss of activity and community structure. Contrastingly, a transfer time during the exponential phase (3 days), favoured stable sequential EtOH production (30mM; 0.35L_{EtOH}/Kg_{waste}), linked to the continuous dominant enrichment of a solventogenic *Clostridium* and an acid-tolerant *Pseudomonas* species.

CRediT authorship contribution statement

PCB: Conceptualization, Data curation, Methodology, Investigation, Visualization, Writing - original draft, Funding acquisition. JD: Writing - review & editing, Supervision. PS: Supervision. NG: Methodology, Writing - review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Sequences from this project have been deposited at DDBJ/EMBL/GenBank under the accession KCKJ00000000. Fermentation products datasets are available at 10.25405/data.ncl.12646388

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Table 1. Maximal soluble fermentation production [mM] in 14-day (NB14dt, B14dt) and 3-day (B3dt) inoculum transfers.

Ferment.product	**NB14dt**			**B14dt**			**B3dt**			
	OI	**Tr1**	**Tr4**	**OI**	**Tr1**	**Tr4**	**OI**	**Tr1**	**Tr3**	**Tr5**
Ethanol	62.05±4.55	18.63±4.99	11.61±2.49	62.96±7.77	25.02±4.03	15.34±1.95	56.85±2.6	33.89±5.20	29.66±3.75	29.90±3.75
Acetic acid	7.42 ±1.99	6.28 ±0.69	4.80 ±0.78	10.75±0.41	4.32 ±1.41	6.34 ±0.35	4.33 ±0.57	3.96 ±1.52	3.72 ±0.99	3.32 ±0.22
Butyric acid	10.63±3.94	2.17 ±0.21	-	5.98 ±0.76	9.49 ±4.18	5.14 ±2.01	20.10±0.91	23.14±5.13	10.39±1.00	19.74±4.80
Formic acid	-	-	-	-	-	-	5.88 ±0.13	5.45 ±1.26	2.60 ±0.28	3.44 ±0.49
Lactic acid	2.60 ±0.84	3.39 ±0.44	3.55 ±0.29	4.1 ±0.38	3.23 ±1.07	3.11 ±0.95	2.06 ±0.04	0.77 ±0.10	1.011±0.10	3.19 ±2.99
Propionic acid	1.47 ±0.06	1.43 ±0.31	-	1.05 ±0.06	0.67 ±0.33	-	1.47 ±0.23	-	-	-
TCarbon meq.	200.36±27.64	55.89± 16.52	49.10± 27.64	197.26± 19.98	99.71± 20.78	74.83± 4.75	200.30±7.47	176.61± 15.83	107.92± 3.04	163.21± 22.59
e- meq.	1101.39± 144.86	383.16± 63.44	232.99± 26.08	1071.41± 114.21	596.76± 104.06	372.36± 24.04	1082.73± 43.56	923.30± 71.30	586.69± 23.35	848.96± 111.11

Ferment. product= Fermentation product. TCarbon meq.= Total carbon soluble fermentation productivity (mM). e- meq.= Estimates of maximal total productivity in terms of electron milliequivalents (e- meq./L). OI= Original Inoculation. Tr*n*= Transfer number *n*.

Figures

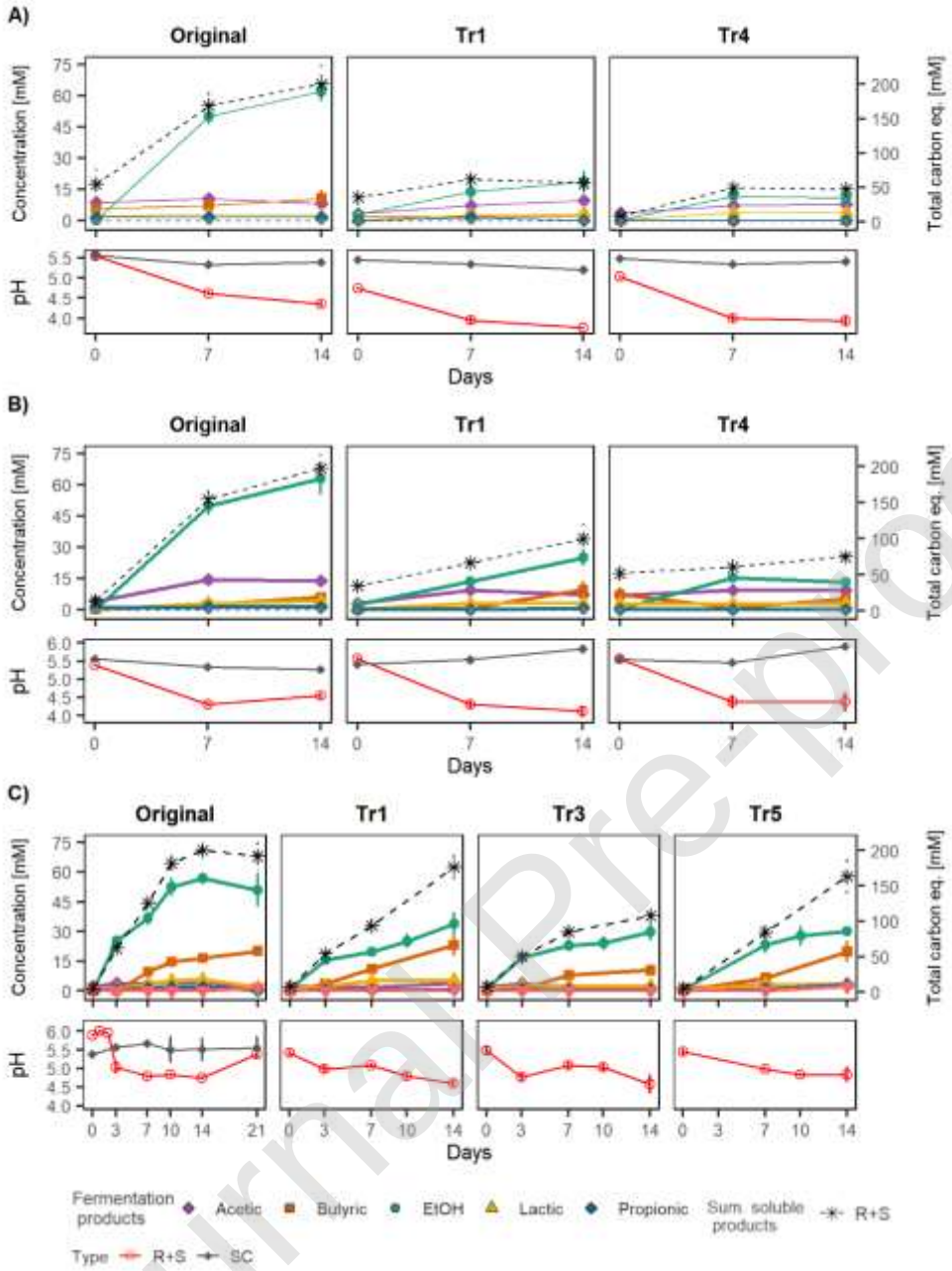

Figure 1. Soluble fermentation products and pH profiles from sequential inoculum transfers experiments. A) NB14dt, B) B14dt, C) B3dt. The sum. soluble products lines represent the total milliequivalents of soluble carbon quantified in R+S inoculated microcosms and substrate-only controls (SC). For the B3dt experiment, SC were only conducted during the OI incubation.

A)

EXP	NB14dt									B14dt									B3dt																
Tr	OI			1			4			OI			1			4			OI						1				3				5		
Days	0	7	14	0	7	14	0	7	14	0	7	14	0	7	14	0	7	14	0	3	7	10	14	21	0	3	7	14	0	3	7	14	0	7	14
S	476	486	160	150	34	29	27	19	21	479	293	221	166	32	30	20	21	24	294	149	125	158	181	139	88	23	24	33	193	156	83	76	90	32	7
D	38.9	3.0	3.3	3.3	2.3	3.1	1.8	1.8	2.0	31.8	4.4	4.8	4.0	1.3	2.4	1.7	1.1	1.7	42.1	4.9	4.4	4.9	4.3	6.6	6.4	1.5	2.2	1.8	35.2	10.5	5.8	7.0	5.7	3.2	3.7
E	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.5

Index

B)

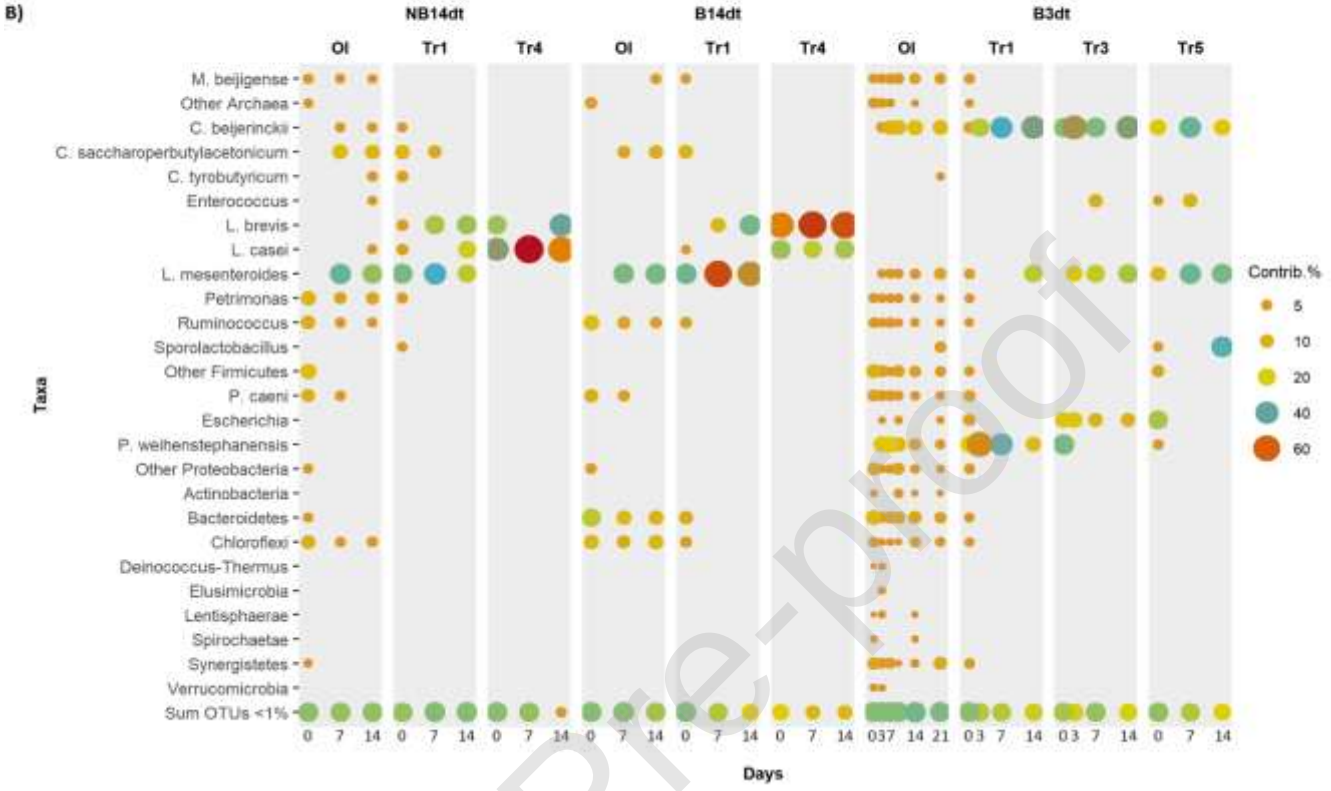

Figure 2. A) Diversity indices of communities enriched at different sampling days across inoculum sequential transfers. B) Average number of individual OTUs contributing to at least 70% of the cumulative similarity in each sampling day x transfer group. The species name is given for OTUs ≥ 99% similar to the said species in multiple GenBank hits, otherwise OTUs contributing to ≥ 1% are grouped into Phyla.

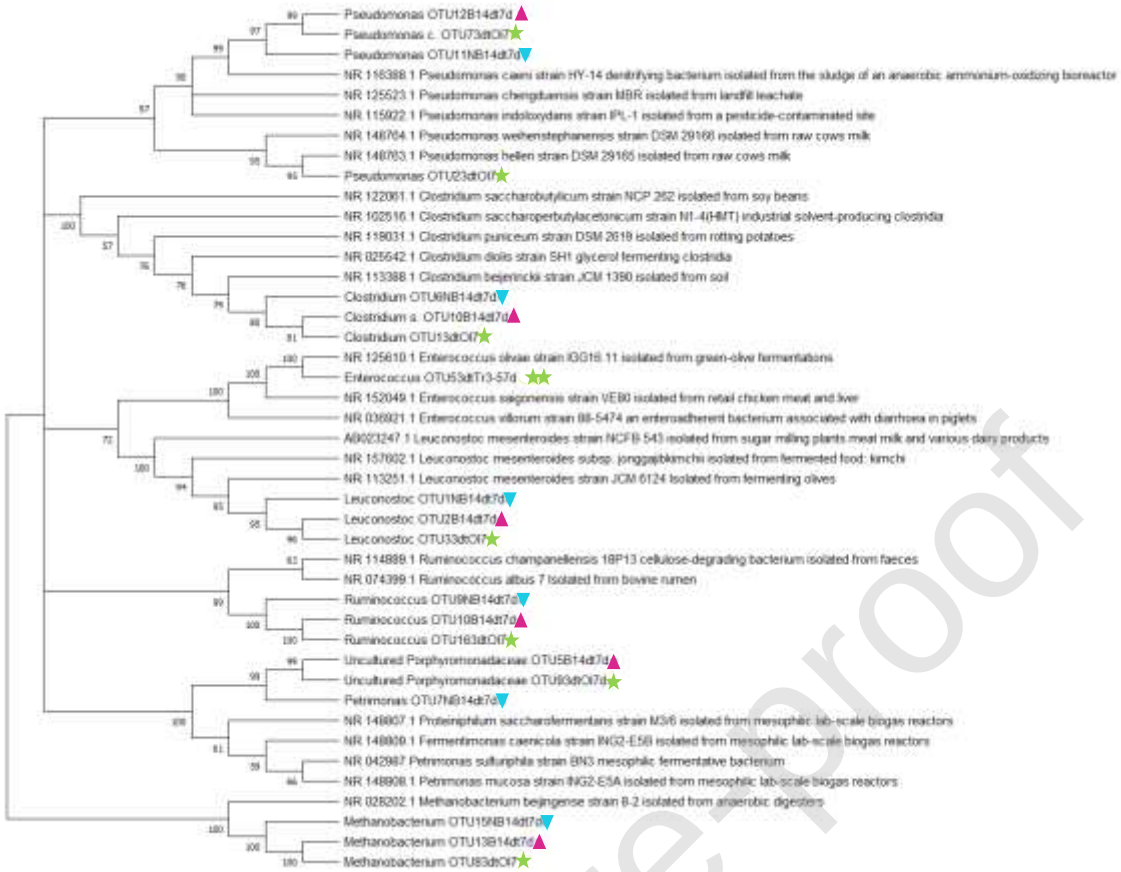

Figure 3. Phylogenetic distance tree (Neighbour-Joining) of key R+S microbial taxa enriched and their close relatives. The tree is based on comparative analysis of selected partial 16S rRNA sequences recovered from three independent experiments (▼=NB14dt, ▲=B14dt and ★= 3dt) at day 7 of their original inoculation batch, with the exception of an Enterococcus related sequence (★★), which was enriched in B3dt experiment at Tr3 and was one of the community members of Tr5 at the last sampling point. The percentage of replicate trees in which the associated taxa clustered together in bootstrap analysis (1000 replicates) are shown next to the branches. The analysis involved 229 nucleotide positions.