**Title: The challenges of monitoring and manipulating anaerobic microbial communities**

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**Abstract**

Mixed anaerobic microbial communities are a key component in valorization of waste biomass via anaerobic digestion. Similar microbial communities are important as soil and animal microbiomes and have played a critical role in shaping the planet as it is today. Understanding how individual species within communities interact with others and their environment is important for improving performance and potential applications of an inherently green technology. Here, the challenges associated with making measurements critical to assessing the status of anaerobic microbial communities are considered. How these measurements could be incorporated into control philosophies and augment the potential of anaerobic microbial communities to produce different and higher value products from waste materials are discussed. The benefits and pitfalls of current genetic and molecular approaches to measuring and manipulating anaerobic microbial communities and the challenges which should be addressed to realise the potential of this exciting technology are explored.

**Keywords:**

**Anaerobic digestion, microbiome, metagenomics, genetic engineering**

# 1. Introduction

Anaerobic microbial communities are ubiquitous in nature, occurring in environments where oxygen is absent. Several types of anaerobic communities such as those found in the gastrointestinal tract of humans are of particular scientific interest, and have been the subject of intense study [(Andrade et al., 2020)](https://paperpile.com/c/Z6n5nF/gBbh) . This review is focused on one such important type of anaerobic community, which is found in the context of anaerobic digestion (AD).

AD is a naturally occurring process through which biomass is progressively broken down in the absence of oxygen to yield an end product primarily consisting of carbon dioxide and methane known as biogas [(Náthia-Neves et al., 2018)](https://paperpile.com/c/Z6n5nF/4YgT). This process is achieved through the concerted action of a complex community of anaerobic microorganisms. Different community members possess complimentary metabolic capabilities, such that the catabolic outputs of certain community members serve as substrates for others [(Batstone et al., 2002)](https://paperpile.com/c/Z6n5nF/vphK). AD is an established approach for recovering resources from waste biomass such as sewage, municipal waste and agri-food residues [(Lim et al., 2020)](https://paperpile.com/c/Z6n5nF/ZCqb). Biogas can be used as a fuel used to generate renewable energy or further upgraded to biomethane as a green replacement for natural gas [(Robson et al., 2017)](https://paperpile.com/c/Z6n5nF/MhWi). More recently the possibility of producing other precursor molecules from AD has also been explored [(Jones et al., 2021; Uludag-Demirer et al., 2019; Wainaina et al., 2019)](https://paperpile.com/c/Z6n5nF/4Kgb%2BSWcB%2B4tCE).

One of the key challenges associated with AD is that unfavourable operating conditions such as feedstock overload can destabilise the community on which the process depends, leading to performance decreases or even in some cases process failure [(Lim et al., 2020)](https://paperpile.com/c/Z6n5nF/ZCqb). This creates a clear incentive to be able to monitor and manipulate the anaerobic microbial communities found in AD. For example, monitoring techniques could be used to detect early warning signals of community destabilisation events, allowing countermeasures to be taken to restabilise the community [(Wu et al., 2019)](https://paperpile.com/c/Z6n5nF/PWh0) and potentially avoid costly downstream process perturbations [(Lim et al., 2020)](https://paperpile.com/c/Z6n5nF/ZCqb). Manipulation could theoretically be used to improve the capabilities of the AD microbial community, for example by increasing its potential for methane production [(Tao et al., 2019)](https://paperpile.com/c/Z6n5nF/F7ab), or by modifying the community to generate greater quantities of valuable products [(Wainaina et al., 2019)](https://paperpile.com/c/Z6n5nF/SWcB). Monitoring and manipulating AD communities is not straightforward. Anaerobic microorganisms in pure culture are difficult to work with in the laboratory since their maintenance is reliant on specialist approaches and equipment [(Mori and Kamagata, 2014)](https://paperpile.com/c/Z6n5nF/MLtM). Working with mixed microbial communities also presents difficulties such as the complexity of understanding such interconnected systems [(Cao et al., 2019)](https://paperpile.com/c/Z6n5nF/NKMv). These challenges will be the main topics considered in the remainder of this review.

# 2. Monitoring

The ability to determine the status of a microbial culture can be highly beneficial to managing the desired outcomes from relevant biotechnological processes. Continuous, real-time monitoring might be ideal, but is not always possible, either because an appropriate technology does not exist, or is too expensive for a particular application. Additional challenges such as fouling of probes or sampling lines may also need to be overcome [(Nguyen et al., 2015)](https://paperpile.com/c/Z6n5nF/3BUt). In-line sampling may allow automated measurements to be performed in a semi-continuous manner, otherwise off-line analysis of samples may be used to provide either close to point-of-sampling, or retrospective insight into the process being monitored. Regular measurements support active management of the process, whereas retrospective analyses may provide quality control data and/or insight into the effect of process variations or interventions.

Current process measurements used to monitor AD processes at scale do not directly assess microbial community dynamics, but rather provide an output that integrates the activities and indirect effects of microbial guilds or consortia in response to the feedstock. A currently incomplete understanding of the biological processes underlying AD means that interpreting the measurements available can be challenging and may lack mechanistic insight. A better understanding of the dynamics of the microbial communities that are associated with AD could lead to more effective tools for monitoring this process [(Amha et al., 2018)](https://paperpile.com/c/Z6n5nF/1nuh). Where in the process off-line samples are sourced from will also influence how these measurements should be interpreted. The material exiting a digester may not accurately represent what is happening in the digester depending on how well mixed the system is and the hydraulic retention time under which it is operating.

## *2.1. Physicochemical process monitoring*

Based on current understanding of the AD process, a number of parameters are routinely monitored. These include gas volume and composition, temperature and pH. Concentrations of methane and carbon dioxide can be readily measured using near infra-red absorbance spectroscopy, other gases such as nitrogen, oxygen, carbon monoxide and hydrogen sulfide can also be measured using electrochemical sensors. Due to stabilisation times, most of these readings are logged semi-continuously (Table 1). More detailed gas analysis is possible, for example, by using gas chromatography and/or mass spectrometry, which provides higher accuracy and sensitivity but at a significantly higher price [(Ward et al., 2011)](https://paperpile.com/c/Z6n5nF/DEtK). These methods can be affected by gas humidity levels (which are generally saturating in AD) so some additional treatment of off-gases may be required.

A wide range of discontinuous off-line measurements based on colorimetric methods are available to monitor a range of physicochemical parameters including alkalinity, chemical oxygen demand, nitrogen, phosphorus, sulfur etc in samples requiring relatively little manipulation prior to measurement (automated systems are available). Volatile fatty acids (VFAs) can be measured colorimetrically, or by using methods such as gas chromatography, which readily separates VFAs for separate quantification. Total and volatile solids are also often quantified in AD samples. Biological Oxygen Demand assays can be used to assess the potential of a given feedstock, but do not accurately reflect the (semi) continuous process of AD at scale (Table 1). Together these measurements provide operators with a series of trends that facilitate an assessment of how stably a process is running without necessarily providing insights into how the process might be optimised, and such measurements can theoretically be incorporated into so-called "soft sensors" (Cruz et al, 2021). Whilst the carbon mass balance can be calculated and assessed to determine how efficient the process is, this does not immediately suggest ways in which the process can be improved. Importantly, the normal operating ranges for a particular set of parameters are context dependent, in that they vary from digester to digester in a feedstock dependent manner and so generalising a control philosophy from such measurements is currently a major challenge.

## *2.2. Discontinuous molecular monitoring*

A range of molecular methods can be used to assess the status of a microbial community. DNA sequencing can be used to catalogue microbial communities using metagenomics methods. 16S rDNA sequencing [(De Vrieze et al., 2018)](https://paperpile.com/c/Z6n5nF/tVe1) and more recently whole metagenome shotgun sequencing have been used extensively to identify members of AD communities. Oxford Nanopore Technologies' "What's In My Pot" (WIMP) workflow provides a more rapid assessment of 16S abundance in communities, but still requires time-consuming DNA extraction and sequencing library preparation techniques before samples can be assessed. An amplification step also potentially introduces a systematic bias into the data collected [(Juul et al., 2015)](https://paperpile.com/c/Z6n5nF/5L7W). Less commonly applied thus far, but also available are metatranscriptomics, metaproteomics [(Hassa et al., 2018; Maus et al., 2020)](https://paperpile.com/c/Z6n5nF/geUQ%2B4QNA) and metabolomics [(Cardona et al., 2020)](https://paperpile.com/c/Z6n5nF/sIkz) as methods that can potentially provide insight into the potential repertoire of metabolisms available in anaerobic digesters and which of these are active when the analysed systems are sampled. While these 'omics are very powerful and can be used to gain insight into the dynamics within AD microbial communities [(Zhu et al., 2020)](https://paperpile.com/c/Z6n5nF/e4PG), such measurements are highly involved, costly in terms of resources, time and expertise and produce large quantities of data that require specialist facilities to analyse and interpret. Thus, these methods are not particularly useful for real time process monitoring, although the insights gained from such studies have the potential to identify important targets against which useful real-time diagnostics could be designed.

*2.3. Microbial-based monitoring*

AD could be monitored through the use of whole cell biosensors incorporating reporter genes, which have been deployed extensively in other biological systems [(van der Meer and Belkin, 2010)](https://paperpile.com/c/Z6n5nF/hhSg). Reporter genes code for a product that has an easily detectable phenotype [(Coralli et al., 2001)](https://paperpile.com/c/Z6n5nF/DqRM), such as the widely used green fluorescent protein (GFP) [(Chudakov et al., 2010)](https://paperpile.com/c/Z6n5nF/XiUS). Reporter gene expression can be linked to a desired stimulus by placing the gene for the chosen reporter under the control of a promoter that is induced (either directly or indirectly) by that stimulus [(Harms et al., 2006)](https://paperpile.com/c/Z6n5nF/eoRc) to generate a proportional response (Figure 1).

Whole cell microbial based monitoring has clear potential in AD since any stimulus of interest in a digester could theoretically be detected by connecting the biological components required to sense and transduce the signal from that stimulus to a suitable reporter gene in an appropriate anaerobic host, provided that these components are known [(Chang et al., 2017)](https://paperpile.com/c/Z6n5nF/Xe75). For example, once early warning indicators for digester destabilising events have been reliably determined, biosensors could be "wired" to detect and report on these indicators, serving as a biological early warning system. Biosensors have several advantages over physicochemical and molecular based AD monitoring techniques in that once engineered they are comparatively cheap and easy to operate, and report on the bioavailable (i.e. biologically relevant) fraction of molecules [(Renella and Giagnoni, 2016; Wu et al., 2019)](https://paperpile.com/c/Z6n5nF/SyIl%2BPWh0). However, a critical limitation of whole cell biosensors in AD is that widely used reporter genes like GFP do not function in anaerobic environments [(Chia et al., 2020, 2019)](https://paperpile.com/c/Z6n5nF/ht8u%2BjoRd). The development of suitable anaerobically compatible reporters is therefore key to aiding the development of whole cell microbial based monitoring systems in anaerobic environments.

### *2.3.1. Visual Reporter Systems*

Visual reporter gene systems such as those based on GFP are crucial biological tools that have been applied for a wide range of purposes across a variety of systems, primarily because of their ease of use [(Chudakov et al., 2010; Ozbakir et al., 2020; Péresse and Gautier, 2019)](https://paperpile.com/c/Z6n5nF/XiUS%2BuYIk%2BIH4f). Currently, no well developed visual reporter system is amenable to use for real time monitoring in strictly anaerobic systems such as AD. Systems based on substrates that are metabolized to coloured products such as the LacZ / beta-galactosidase assay are reliant on cell lysis [(Gutiérrez et al., 2015)](https://paperpile.com/c/Z6n5nF/nGMw), which would necessitate digester sampling and laboratory analysis, making them unsuitable for real time monitoring. Other visual reporters such as luminescence systems using luciferase genes or fluorescence methods based on GFP and its derivatives are not usable in AD, because they require oxygen to function [(Gutiérrez et al., 2015; Ozbakir et al., 2020)](https://paperpile.com/c/Z6n5nF/nGMw%2BuYIk).

The incompatibility of visual reporter systems with anaerobic environments such as AD has driven the recent development of several systems based on fluorescence that do work anaerobically. The majority of these systems consist of two parts: a protein component capable of binding to a fluorophore and the fluorophore itself, which is responsible for fluorescence [(Péresse and Gautier, 2019)](https://paperpile.com/c/Z6n5nF/IH4f). This is in contrast to GFP based systems in which the GFP protein alone comprises both the protein and fluorophore moieties [(Chudakov et al., 2010)](https://paperpile.com/c/Z6n5nF/XiUS).

Flavin-mononucleotide based fluorescent proteins (FbFPs) were among the first developed anaerobically compatible fluorescent reporter systems. These were generated by engineering light oxygen voltage (LOV) domain proteins, resulting in considerably increased fluorescence while bound to their flavin mononucleotide (FMN) fluorophores [(Drepper et al., 2007)](https://paperpile.com/c/Z6n5nF/ahH6). An intrinsic advantage of FbFP systems is their fluorophores are readily available within most cells [(Buckley et al., 2015)](https://paperpile.com/c/Z6n5nF/Wtzy), avoiding the requirement to add these factors exogenously. However, FbFPs have two major drawbacks which limit their utility as reporters in anaerobic environments. Firstly, FbFPs are substantially dimmer than GFP. Secondly, FbFPs emit light at similar wavelengths to that of cellular autofluorescence, making it harder to distinguish their (already relatively dim) visual signal from background fluorescence [(Chia et al., 2019)](https://paperpile.com/c/Z6n5nF/ht8u).

Several more recently developed anaerobically compatible fluorescent reporters such as the “self-labelling” SNAP-tag and Halo-tag systems are brighter than FbFPs [(Chia et al., 2019)](https://paperpile.com/c/Z6n5nF/ht8u). Other alternatives, including the fluorescence-activating and absorption shifting tag (FAST) system and bilin-binding proteins have been further developed to enable anaerobic two-colour reporting [(Chia et al., 2020; Tebo et al., 2020)](https://paperpile.com/c/Z6n5nF/mPGL%2BjoRd). While all of these systems have been demonstrated to function in strict anaerobes, their drawback is that unlike FbFPs their ligands are not produced by cells, so must be exogenously supplied [(Charubin et al., 2020; Chia et al., 2020, 2019; Streett et al., 2019)](https://paperpile.com/c/Z6n5nF/ht8u%2BjoRd%2BtO1k%2B5tY7). This would likely be impractical in AD in an industrial context since this would either necessitate the supply of substantial amounts of these fluorophores to whole digesters, which would almost certainly be financially prohibitive, or require sampling approaches, which are not suitable for real time monitoring.

Alternative approaches for visual reporting compatible with oxygen-free environments, such as RNA or DNA based aptamers capable of binding to oxygen independent fluorophores, or the use of unnatural fluorescent amino acids in engineered organisms with an expanded genetic code, are at different stages of development and have been reviewed elsewhere [(Chia et al., 2019)](https://paperpile.com/c/Z6n5nF/ht8u).

 One key consideration for the future of visual reporters in AD is that even if an “ideal” visual reporter suitable for real time reporting in AD (i.e. that produces a strong, clean signal without the need for supply of exogenous chemicals) were to be developed, it would potentially still be of limited value in an AD environment since the contents of digesters are non-transparent [(Del Valle et al., 2020a)](https://paperpile.com/c/Z6n5nF/vFYn).

### *2.3.2. Volatile-based Reporter Systems*

An alternative to visual reporters that may be more amenable to anaerobic environments is volatile-based reporting. Volatile reporting systems work through the exogenous expression of a volatile-producing enzyme followed by detection of the resulting volatile through gas chromatography mass spectrometry (GC-MS) [(Cheng et al., 2016)](https://paperpile.com/c/Z6n5nF/te2z). Volatile reporters have been used in a number of proof-of-principle studies including monitoring horizontal gene transfer between bacteria [(Cheng et al., 2016)](https://paperpile.com/c/Z6n5nF/te2z), detection of mercury contamination in soil [(Liu et al., 2020)](https://paperpile.com/c/Z6n5nF/kUO5), and investigating the effect of soil properties on the bioavailability of plant signalling molecules involved in plant / microbe signalling [(Del Valle et al., 2020b)](https://paperpile.com/c/Z6n5nF/PSMD).

Since their initial development, volatile reporting systems have undergone a number of refinements. Cheng et al. (2018) developed a system to measure inducer levels with greater accuracy by engineering bacteria containing a pair of volatile producing enzymes. In this study, bacteria engineered to generate a methyl bromide producing enzyme under the control of a promoter induced by the quorum sensing molecule acylhomoserine lactone (AHL) were able to report on the presence of AHL. The inclusion of a second constitutively-expressed ethylene forming enzyme (EFE) allowed quantitative reporting of AHL levels by enabling the methyl bromide signal (produced only in response to AHL) to be normalised to ethylene levels (produced constantly by all biosensor cells) [(Cheng et al., 2018)](https://paperpile.com/c/Z6n5nF/rVmE). A volatile-based logic gate was created by splitting the gene encoding a volatile producing enzyme into two units tethered to linker peptides, each under the control of a different inducible promoter. Only when both inducers were present were the two units expressed and a functional enzyme generated, resulting in production of the reporter volatile [(Fulk et al., 2020)](https://paperpile.com/c/Z6n5nF/eRac).

Volatile reporting has clear potential in AD as it would not be subject to the same limitations which make visual reporters difficult to use in this environment. An added advantage is that volatile signals could easily be collected from the anaerobic digester gas outlet, enabling monitoring to be conducted in a truly non-disruptive manner (Figure 2). While the ability to produce a number of different volatiles has been engineered into microbes, to be usable in AD, volatile producing enzymes must fit two additional parameters. Firstly, they must be capable of functioning in an anaerobic host organism. As it stands, only a single volatile producing enzyme/volatile pair (methyl halide transferase producing methyl bromide) has been robustly demonstrated to function anaerobically [(Cheng et al., 2016)](https://paperpile.com/c/Z6n5nF/te2z). Other volatiles which have previously been used in volatile reporting such as ethylene would not be suitable because all known biological pathways for its synthesis require oxygen [(Eckert et al., 2014; Fukuda et al., 1992; Mansouri and Bunch, 1989)](https://paperpile.com/c/Z6n5nF/Qw7L%2Bz2gi%2BEpdu). Since the normalisation method for more accurate determination of inducer concentration requires two distinct volatiles [(Cheng et al., 2018)](https://paperpile.com/c/Z6n5nF/rVmE), this creates a clear incentive for the identification of additional volatiles suitable for production in anaerobic conditions. Secondly, the volatile product generated by the chosen enzyme must be otherwise absent (or near absent) in the headspace of a typical anaerobic digester to enable the signal generated to be distinguishable from background levels. For example, while genetic engineering for the production of isoprene has been explored extensively in bacteria [(Ye et al., 2016)](https://paperpile.com/c/Z6n5nF/4LGs) and more recently anaerobically in archaea [(Aldridge et al., 2021)](https://paperpile.com/c/Z6n5nF/CbFZ), since most bacteria are thought to possess at least one endogenous isoprene synthesis pathway [(Lee et al., 2020)](https://paperpile.com/c/Z6n5nF/i8AW) this volatile is likely to be present at (relatively) high concentrations in AD headspace, making it an unsuitable choice for volatile reporting applications.

The possibile loss through sorption of the produced volatile product to material present in the digester, or through uptake / degradation by other members of the anaerobic microbial community is also an important consideration [(Del Valle et al., 2020a)](https://paperpile.com/c/Z6n5nF/vFYn). These potential issues have yet to be investigated in the context of AD, and would be worthwhile avenues of exploration for future research.

### *2.3.3. Other reporter systems and alternatives*

Several other genetically encoded reporter systems, which may be suitable for use in whole cell AD biosensors such as ice nucleation proteins and gas vesicles are available [(Del Valle et al., 2020a)](https://paperpile.com/c/Z6n5nF/vFYn), but such techniques will likely require further development before deployment in an industrially useful manner in AD. Whole-cell bioelectrochemical biosensors are another alternative that are not dependent on reporter gene technologies to function since the electrical signals they produce can be measured directly using electrodes [(Roggo and van der Meer, 2017)](https://paperpile.com/c/Z6n5nF/7qZ7). These systems have been of great interest in the monitoring of AD metabolites such as VFAs. To date most of these systems have been tested only in laboratory or pilot scale situations [(Sun et al., 2018)](https://paperpile.com/c/Z6n5nF/oVQu). A notable exception is the biofilm-based Sentry system offered by Island Water Technologies. While these systems clearly have great potential they are currently hampered by issues around sensitivity, stability and the ability to directly relate measurements to a mechanistic understanding of mixed microbial community biology. The potential utility of bioelectrochemical systems for AD monitoring has been reviewed in detail recently [(Singh and Kumar, 2021)](https://paperpile.com/c/Z6n5nF/5CEN).

# 3. Manipulating anaerobic microbial communities

The ability to reproducibly produce the same or similar outcomes from single species microbial batch monocultures has a long and well established history [(Rosenberg et al., 1987; Studier et al., 1990)](https://paperpile.com/c/Z6n5nF/sL5k%2B3hMO). The controlled production of recombinant protein by the addition of an inducing molecule is used routinely [(Studier et al., 1990; Studier and Moffatt, 1986)](https://paperpile.com/c/Z6n5nF/HR2I%2B3hMO). Such additions are often based on monitoring parameters such as optical density or dissolved oxygen. Autoinduction of similar responses by genetically modified organisms [(Studier, 2005)](https://paperpile.com/c/Z6n5nF/Sqwy) can mimic the biological integration of measurement and response to changing environmental conditions. Understanding these responses can be used to manipulate the microorganism into a desired outcome. At present, the ability to predict the outcomes of an external intervention on a mixed microbial community is hampered by a lack of understanding of the interplay between different species in such communities. Consequently, manipulating an anaerobic microbial community to change, and ideally improve the outcome of a process such as AD is a substantial challenge.

## *3.1. Operational parameters that could modify community composition*

The AD process can be influenced by many operational factors that affect the outcome of digestion. Feedstocks play a central role in AD microbial community responses. Substrate composition will have great influence over which organisms thrive with effects potentially cascading through the trophic layers from those responsible for primary decomposition of polymers through hydrolysis down to methanogenic archaea, which can produce methane from a variety of small molecules [(Costa and Leigh, 2014; Zhu et al., 2020)](https://paperpile.com/c/Z6n5nF/s6vn%2Be4PG). The source and availability of this material also has implications for AD community composition: more variable feedstocks will promote a community with a broader metabolic repertoire [(Fernandez-Bayo et al., 2020)](https://paperpile.com/c/Z6n5nF/ooUc), whereas more consistent feedstocks potentially lead to community specialisation and extinction of redundant species, which may result in a better-performing community that is less resilient to changes. Due to the non-sterile nature of most feedstocks, storage of even consistent feedstocks is likely to result in compositional changes that could cause AD community changes and impact process performance (Table 2).

Pretreatment of feedstocks [(Millati et al., 2020)](https://paperpile.com/c/Z6n5nF/RF77) may affect AD communities. Pasteurization, thermal hydrolysis [(Tong et al., 2019; Zhang et al., 2021)](https://paperpile.com/c/Z6n5nF/QCZl%2BnXvb), CAMBI [(Westerholm et al., 2019)](https://paperpile.com/c/Z6n5nF/QUbx), microwave [(Kor-Bicakci et al., 2020)](https://paperpile.com/c/Z6n5nF/TYZ8), and enzymatic pretreatments seek to produce a more "digestible" feed in which substrates are more easily accessible to microbial species, resulting in reduced digestion times and/or higher gas yields, or safer, more inert digestates that are easier to dispose [(Yin et al., 2020)](https://paperpile.com/c/Z6n5nF/Ql5q). Such treatments also produce raised available nitrogen levels, which can influence alkalinity and VFA levels [(Jiang et al., 2019; Yirong et al., 2017)](https://paperpile.com/c/Z6n5nF/805M%2BKtQV), which then further influence the microbial community [(B. Li et al., 2021)](https://paperpile.com/c/Z6n5nF/xIwl). Similarly, effects can be expected from blending feedstocks (such as the co-digestion of lignocellulosic material and sewage sludge), aimed at providing better C/N ratios for optimal gas production [(Fernandez-Bayo et al., 2020)](https://paperpile.com/c/Z6n5nF/ooUc). In addition to an important role for trace element additions (particularly in food waste AD [(Banks et al., 2012; Zhang and Jahng, 2012)](https://paperpile.com/c/Z6n5nF/Z3aO%2BqFYZ)), numerous commercial additives are available that aim to enhance community activity through a variety of mechanisms that are likely to influence microbial dynamics [(Arif et al., 2018; Barua and Dhar, 2017; Rasapoor et al., 2020; Romero-Güiza et al., 2016)](https://paperpile.com/c/Z6n5nF/eoyQ%2BQVXn%2BMW62%2BvLe1).

Mechanical and chemical engineering interventions can be used to manipulate AD microbial communities. Physical separation of different stages of the AD process could potentially result in a more efficient process [(Gómez Camacho et al., 2019)](https://paperpile.com/c/Z6n5nF/yXjg). Upflow Anaerobic Sludge Blanket (UASB) reactors produce structured granules that contain concentric rings of different species of microbes [(Gagliano et al., 2020; Nordgård et al., 2018; Trego et al., 2020)](https://paperpile.com/c/Z6n5nF/rHSj%2B52we%2BTWis). Operating temperature [(Li et al., 2015)](https://paperpile.com/c/Z6n5nF/9ZWi), hydraulic retention time, effectiveness of mixing [(McLeod et al., 2019)](https://paperpile.com/c/Z6n5nF/pjNf), gas recirculation and length of intervals between feeding could all result in (potentially predictable) AD community changes that could be used to manipulate the microbial response.

While all these factors might be used to manipulate the AD process, they also present potential pitfalls. Feedstock consistency can be difficult to realise due to supply line challenges. Additional facilities for composition testing and blending may be required. Seasonal variations might cause unavoidable feedstock differences [(Resende et al., 2016)](https://paperpile.com/c/Z6n5nF/hpc6). The CAPEX and/or OPEX costs of any intervention need to be weighed against the potential increase in returns as well as the improved productivity and efficiency of the process. Whether this affects process resilience should also be considered. Ultimately the ability to use any of these parameters to deliberately manipulate the AD microbial community relies on a better understanding of cause and effect. In turn this requires measurements that can be used to build hypotheses and models that provide mechanistic insight into how microbes in anaerobic digesters respond, both as individuals and as a community, to environmental changes.

## *3.2. Direct genetic manipulation*

Genetic engineering is a well established approach that could be used to manipulate organisms within AD microbial communities. This technique involves the introduction of user-determined DNA sequences into an organism of choice, with the aim of enabling it to perform some useful function [(Atomi et al., 2012)](https://paperpile.com/c/Z6n5nF/FerR). Genetic engineering facilitates manipulations that would be impossible to achieve through evolutionary approaches, such as imparting entirely novel abilities upon an organism [(Cao et al., 2019)](https://paperpile.com/c/Z6n5nF/NKMv). DNA sequences capable of imparting the desired functionality can be introduced either on a plasmid, which replicates within the cell, or by integration onto the host chromosome. The latter is often preferable since it is more stable and does not necessitate the use of continuous selection [(Saleski et al., 2021)](https://paperpile.com/c/Z6n5nF/8lke).

The ability to genetically engineer a given organism is dependent on the availability of genetic tools for that organism, including for example selectable markers, promoters and terminators, ribosome binding sites and reporter gene systems [(Riley and Guss, 2021)](https://paperpile.com/c/Z6n5nF/HZYk). These tools generally need to be developed separately for different organisms [(Waller et al., 2017)](https://paperpile.com/c/Z6n5nF/0V7e). Tool development can be extremely laborious and time consuming [(Riley and Guss, 2021)](https://paperpile.com/c/Z6n5nF/HZYk), effectively limiting the majority of genetic engineering efforts to organisms for which genetic tools are already available. Genetic tools are available in several anaerobic species known to be present in AD, such as members of the *Clostridium* [(Gyulev et al., 2018)](https://paperpile.com/c/Z6n5nF/K6Rl), *Bacteroides* [(García-Bayona and Comstock, 2019)](https://paperpile.com/c/Z6n5nF/TfoV), and methanogens [(Atomi et al., 2012)](https://paperpile.com/c/Z6n5nF/FerR). Genetic modification could be used to augment the abilities of genetically tractable anaerobic microorganisms, which could be used to improve the performance of the microbial communities to which they belong. Direct manipulation of genetically tractable microorganisms known to be present in AD has been used to introduce novel metabolic capabilities in pure culture. For example, *Methanococcus maripaludis* has been engineered to produce the high value compound geraniol [(Lyu et al., 2016)](https://paperpile.com/c/Z6n5nF/lsK1), and *Methanosarcina acetivorans* and *Methanosarcina barkeri* were engineered to produce isoprene [(Aldridge et al., 2021)](https://paperpile.com/c/Z6n5nF/CbFZ). Demonstrations of the effectiveness of competitiveness of engineered organisms within AD communities rather than in monocultures currently appear to be lacking.

Genetic engineering is suitable for the generation of completely novel phenotypes [(Cao et al., 2019)](https://paperpile.com/c/Z6n5nF/NKMv). However, this approach also comes with the distinct drawback that anaerobic microorganisms manipulated in this manner are also classified as genetically modified organisms (GMOs), so their use would likely be subject to regulatory barriers [(Si et al., 2017)](https://paperpile.com/c/Z6n5nF/emSj).

## *3.3. Directed evolution based manipulation*

Directed evolution can be used to manipulate anaerobic microbial communities. This approach uses natural selection to evolve improvements into microorganisms. For example, a population of cells can be evolved for greater performance under a chosen condition by exposing them to that condition. Mutations conferring a phenotypic advantage in the chosen condition are selected for and generally become fixed, generating a fitter population of cells. Through prolonged exposure this cycle can be repeated many times to generate a population of cells that is substantially fitter with respect to the condition it has been evolved to than the original population [(McDonald, 2019; Si et al., 2017; Van den Bergh et al., 2018)](https://paperpile.com/c/Z6n5nF/emSj%2BIttu%2BR08i). Directed evolution is best suited to augmenting abilities that already exist within the population of cells being modified [(Cao et al., 2019)](https://paperpile.com/c/Z6n5nF/NKMv), but is generally not suitable for imparting entirely new abilities upon cells. It should be noted that a combination of genetic engineering to introduce some new characteristic into a microorganism followed by subsequent directed evolution to improve the performance of that organism can be used [(McDonald, 2019)](https://paperpile.com/c/Z6n5nF/R08i)

Interesting examples of directed evolution in anaerobic microbial communities include the development of increased tolerance of an AD community to co-digested toxic byproducts of lignocellulosic pyrolysis. The resulting community could withstand higher quantities of these toxins without significant process inhibition, although the relative contribution of changes at the DNA level within community members compared with changes in community composition itself to this community phenotype was not determined [(Zhou et al., 2019)](https://paperpile.com/c/Z6n5nF/VR0f). Separately, directed evolution was used to increase the fitness of a facultative anaerobe under anaerobic conditions. After 2000 generations of directed evolution to an anaerobic environment, *E. coli* populations were demonstrated to be around 40% fitter under anaerobic conditions than the ancestral strain [(Finn et al., 2017)](https://paperpile.com/c/Z6n5nF/VNy2). Directed evolution has even been used to generate strains of anaerobic bacteria with repurposed oxygen reducing machinery. The sulphate reducing bacterium *Desulfovibrio vulgaris*, naturally capable of using its endogenous oxygen reducing machinery to protect against transient oxygen exposure, was experimentally evolved to use a modified version of this machinery for energy generation [(Schoeffler et al., 2019)](https://paperpile.com/c/Z6n5nF/BKX2).

The main advantages of directed evolution for the manipulation of AD microbial communities include a lack of reliance on genetic tools or prior knowledge concerning the mechanisms that underlie the desired phenotypes in the organism(s) of choice [(Si et al., 2017)](https://paperpile.com/c/Z6n5nF/emSj). In principle, directed evolution can also be used to evolve different members of a community together [(Lawrence et al., 2012)](https://paperpile.com/c/Z6n5nF/VcAH). A key limitation of directed evolution for community manipulation is that the genetic changes, which underlie increases in fitness in the condition used to impose the selective pressure, may come at the cost of unwanted phenotypic effects elsewhere, which may make the population less fit with respect to other parameters [(Van den Bergh et al., 2018)](https://paperpile.com/c/Z6n5nF/Ittu). While this approach will generally yield organisms with an increased fitness with respect to the condition they have been evolved to, exactly how this is achieved cannot be controlled, which may lead to unpredictable outcomes [(McDonald, 2019)](https://paperpile.com/c/Z6n5nF/R08i).

## *3.4. Community composition manipulation*

Microbial community manipulation can be achieved by creating an entirely synthetic community from scratch by co-culturing a set of pre-selected microorganisms [(Grosskopf and Soyer, 2014)](https://paperpile.com/c/Z6n5nF/eXAm). This approach could be used to select the “best” microorganisms for the job (i.e. exhibiting desirable characteristics that would be anticipated to work well together as a community) [(Johns et al., 2016)](https://paperpile.com/c/Z6n5nF/iQks) while simultaneously excluding other undesirable options [(Diender et al., 2021)](https://paperpile.com/c/Z6n5nF/Eeac). This approach could be particularly useful for AD since one of the main causes of fluctuations in process performance is community instability [(Lim et al., 2020)](https://paperpile.com/c/Z6n5nF/ZCqb). A simplified pre-optimised AD community might be more stable, and this stability could be further enhanced by engineering each community member to be auxotrophic for a certain metabolite to promote interdependence [(Johns et al., 2016)](https://paperpile.com/c/Z6n5nF/iQks). Highly simplified synthetic communities have already been produced in AD to investigate interspecies interactions [(Chen et al., 2019)](https://paperpile.com/c/Z6n5nF/BkZP).

This approach theoretically permits a higher level of control over community composition. Key challenges include maintaining purity of the constructed community by controlling the influx of unwanted organisms, as well as preventing the mutational loss of genetic machinery in the case of engineered community members [(Johns et al., 2016)](https://paperpile.com/c/Z6n5nF/iQks). An alternative approach to community composition manipulation could be to add wild-type or engineered organism(s) to a pre-existing microbial community [(Cao et al., 2019)](https://paperpile.com/c/Z6n5nF/NKMv). AD studies have investigated the utility of bioaugmentation using live microorganisms to improve process performance, with varying and seemingly unpredictable levels of success, as discussed previously [(De Vrieze and Verstraete, 2016)](https://paperpile.com/c/Z6n5nF/lIv9). Both these approaches are currently limited by an incomplete understanding of AD communities. Knowledge of the relative roles of their various members [(Venkiteshwaran et al., 2015)](https://paperpile.com/c/Z6n5nF/8stv) may not be sufficient to enable the most appropriate candidate organisms to be reliably identified.

## *3.5. Emerging methods for community manipulation*

*3.5.1. CRISPR based methods*

CRISPR is perhaps best known for its engineered derivative systems such as CRISPR/Cas9, which has been used extensively as a genetic engineering tool. The endogenous function of CRISPR systems provides a type of adaptive immunity in bacteria and archaea, and both endogenous and exogenously introduced CRISPR systems can be used as an alternative method to manipulate microbial communities [(Ramachandran and Bikard, 2019)](https://paperpile.com/c/Z6n5nF/3HPW).

One approach involves the use of CRISPR systems to selectively destroy specific microbial community members [(Ramachandran and Bikard, 2019)](https://paperpile.com/c/Z6n5nF/3HPW). For example, CRISPRs were designed to selectively remove either of two *E. coli* strains that were >99% similar at the sequence level [(Gomaa et al., 2014)](https://paperpile.com/c/Z6n5nF/R7QG). Broader community targeting (e.g. multiple species belonging to the same genus) can be achieved by modifying the targeting specificity of the CRISPR system to some genetic feature common to members of that genus. Another approach is to augment the native CRISPR function of existing microbial community members by “immunising” them against certain phages [(Barrangou and Notebaart, 2019)](https://paperpile.com/c/Z6n5nF/F27X). A similar approach based on the introduction of exogenous CRISPR systems has previously been demonstrated [(Bikard et al., 2014)](https://paperpile.com/c/Z6n5nF/QKWE).

These techniques have clear potential as community manipulation tools in AD systems. Immunisation techniques using endogenous CRISPR systems should be theoretically possible in most archaea since the majority are known to possess endogenous CRISPR systems [(Li and Peng, 2019)](https://paperpile.com/c/Z6n5nF/WBUK). This approach could be particularly valuable in methanogens, which are generally understood to be the most vulnerable members of the AD consortium [(Lim et al., 2020)](https://paperpile.com/c/Z6n5nF/ZCqb). Community manipulations using CRISPR based approaches would benefit from the versatile targeting capabilities of these systems [(Shabbir et al., 2019)](https://paperpile.com/c/Z6n5nF/FxAv), and high specificity compared to other selection methods such as antibiotics [(Gomaa et al., 2014)](https://paperpile.com/c/Z6n5nF/R7QG). Delivery of the CRISPR system into microbial community members commonly relies on phage based delivery methods, and while approaches to tune the breadth of phage targeting and non-phage alternative delivery methods are being explored [(Fagen et al., 2017)](https://paperpile.com/c/Z6n5nF/axq6), existing options may not be capable of delivering the CRISPR system to a large enough subset of the AD community to be useful.

*3.5.2. Quorum sensing*

Quorum sensing (QS) is a form of communication used by microorganisms based on the ability to synthesise and detect signalling molecules that are able to cause changes in community gene expression once present at a certain concentration [(De Vrieze and Verstraete, 2016)](https://paperpile.com/c/Z6n5nF/lIv9). QS represents a system through which microorganisms can modulate their behaviour in response to cell densities, enabling microbial communities to respond in a concerted manner to changes in their surroundings [(Zhao et al., 2021)](https://paperpile.com/c/Z6n5nF/qrVW). Since QS is involved in community communication and function [(Zhao et al., 2021)](https://paperpile.com/c/Z6n5nF/qrVW), the ability to manipulate QS could in turn be used to manipulate communities.

QS manipulation using genetic approaches has been demonstrated in monocultures, however, work in this field is increasingly examining the manipulation of these systems in mixed microbial communities, and has been thoroughly reviewed recently [(Stephens and Bentley, 2020)](https://paperpile.com/c/Z6n5nF/jf0u). In one particularly striking example of the potential power of this approach for community manipulation, *E. coli* cells were programmed to produce antimicrobial peptides and a self lysis factor in response to quorum sensing signals produced by pathogenic *Pseudomonas aeruginosa*. The result was a strain of *E. coli* that was effectively able to “self-destruct” in response to the presence of *P. aeruginosa*, releasing the antimicrobial peptides triggering cell death of the pathogen [(Saeidi et al., 2011)](https://paperpile.com/c/Z6n5nF/n6dj). If adapted for use in AD communities, such a system could potentially remove undesirable community members.

There is increasing evidence that endogenous QS systems are widespread in AD communities, as demonstrated by recent studies [(J. Li et al., 2021; Zhang et al., 2019)](https://paperpile.com/c/Z6n5nF/gWoP%2BlRPr). There are also examples where QS based community manipulation has been attempted in AD. The addition of a quorum quenching enzyme, which selectively cleaves QS signalling molecules from Gram negative bacteria, to a digester resulted in a fall in pH, changes in community composition and a marked decrease in methane production. The authors suggested that the disruption of signalling between Gram negative AD community members may have led to a decrease in production of antimicrobials against their competitors, resulting in a rise in acid producing Gram positive community members and therefore a drop in pH, which inhibited methanogenesis [(Nguyen et al., 2019)](https://paperpile.com/c/Z6n5nF/zQPV). The introduction of an *E. coli* strain engineered to remove quorum sensing molecules from an anaerobic bioreactor during recovery from induced organic toxic shock resulted in improved reactor recovery, which the authors suggest may have been due to changes in the bacterial community composition as a result of loss of the quorum sensing molecule [(Xiao et al., 2019)](https://paperpile.com/c/Z6n5nF/DyVh). While these studies represent an initial step into the use of QS to manipulate AD communities, progress with this approach may be limited by gaps in current understanding of exactly how these systems work in AD [(De Vrieze and Verstraete, 2016)](https://paperpile.com/c/Z6n5nF/lIv9).

*3.5.3. Phage*

Phage are known to play a role in AD community dynamics since their life cycle involves the infection and subsequent destruction of community members [(Heyer et al., 2019; Junyu Zhang et al., 2017)](https://paperpile.com/c/Z6n5nF/NkRy%2BJDhD). Phage present an additional avenue to AD community manipulation. Approaches for potentially useful phage mediated manipulations of microbial communities in wastewater treatment processes, as well as potential drawbacks of phage based approaches have previously been reviewed [(Withey et al., 2005)](https://paperpile.com/c/Z6n5nF/77Ae). Possible applications include the selective removal of pathogenic community members from sludge, or the targeted killing of undesirable AD community members that reduce methane yields by competing with other members such as methanogens for substrates [(Withey et al., 2005)](https://paperpile.com/c/Z6n5nF/77Ae).

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# 6. Areas for future research

A major challenge for both monitoring and manipulation of anaerobic microbial communities is a lack of understanding of the species-to-species interactions that result in the collective behaviour of the system. While some syntrophies have been well mapped under specific conditions (particularly at lab scale) [(Zhu et al., 2020)](https://paperpile.com/c/Z6n5nF/e4PG), a better understanding of how an integrated response is generated from these disparate interactions under process conditions would allow useful targets to be identified for both monitoring and manipulation of these communities.

Another factor, which has limited progress in engineered whole cell approaches for AD community monitoring and manipulation, is the limited anaerobically compatible reporter gene toolbox. Recent advances in this area appear promising [(Chia et al., 2019)](https://paperpile.com/c/Z6n5nF/ht8u), and continued development should provide a set of robust, continuous, real-time monitoring tools suitable for use in anaerobic environments.

Survival could limit the utility of any organism introduced into an existing microbial community for the purposes of monitoring or manipulation. For an introduced microorganism to be able to invade and persist within the target community it must be able to compete with endogenous community members [(Bosch et al., 2013; Litchman, 2010)](https://paperpile.com/c/Z6n5nF/gi4k%2BUSJq), since failure to do so would result in its loss from the system and subsequent inability to carry out its intended function [(Perpetuo et al., 2011)](https://paperpile.com/c/Z6n5nF/qL8m). Competition between endogenous community members is known to be an important factor in AD [(Jiang et al., 2021)](https://paperpile.com/c/Z6n5nF/skgK), but current understanding of how such communities could be invaded so that one species might displace another requires more research and consideration of the differences between open and closed systems (e.g. a traditional wastewater treatment system constantly introduces new microbes, whereas some pretreatments such as thermal hydrolysis are designed to lyse microbial cells) [(Pholchan et al., 2013; Sierocinski et al., 2017)](https://paperpile.com/c/Z6n5nF/Erxa%2Bf9M7). The challenge of introducing desired microorganisms into AD communities could be even more severe for GMOs [(Amha et al., 2018)](https://paperpile.com/c/Z6n5nF/1nuh), since they are generally considered to be less competitive than their wild-type counterparts [(Lenski, 1993)](https://paperpile.com/c/Z6n5nF/DTGa), and the metabolic burden of expressing introduced gene products [(Roell et al., 2019)](https://paperpile.com/c/Z6n5nF/nWNH) could further reduce competitiveness. By using AD endogenous microorganisms as engineering chassis this problem could be to some extent addressed as they may be expected to be more competitive in their native environment. The creation of an additional niche within the community, which only the engineered organism can exploit, is another possible approach (Figure 3) as has been demonstrated in mouse gut models [(Shepherd et al., 2018)](https://paperpile.com/c/Z6n5nF/q8X1). Investigating the applicability of these and other strategies to improve the survival of organisms introduced into anaerobic digesters is an important avenue for future research.

Environmental and ethical concerns regarding potential ecosystem damage, which could be caused by the escape and proliferation of genetically modified microorganisms in natural communities, or through transmission of the modified DNA they carry to wild microbial community members through horizontal gene transfer [(Del Valle et al., 2020a; Prakash et al., 2011)](https://paperpile.com/c/Z6n5nF/qbeJ%2BvFYn), are likely to present a barrier to the deployment of GMOs in real world AD community monitoring and manipulation scenarios. Biocontainment methods, which incorporate a genetically programmed lethality phenotype triggered by host organisms entering an environment which they should not occupy, could offer a solution to this problem. Different biocontainment designs have been investigated [(Kim and Lee, 2020)](https://paperpile.com/c/Z6n5nF/rrxn). For example, engineered kill switches were developed by introducing genetic circuitry into microorganisms capable of synthesising a lethal toxin. Constant addition of a specific combination of small molecule inducers is required to prevent toxin gene expression, such that organisms, which enter an environment where the correct combination of inducers is not provided, synthesise the toxin and die [(Chan et al., 2015)](https://paperpile.com/c/Z6n5nF/WCj5). In another study, a plasmid carrying a gene of interest which had been physically entangled with a toxin gene transcribed from a different reading frame was introduced into an *E. coli* conjugation donor strain which also expressed an antitoxin capable of neutralising the toxic product. The system was shown to substantially reduce movement of the gene of interest into other *E. coli* through horizontal gene transfer since they were generally unable to receive the gene of interest without the toxin gene, but lacked the ability to inactivate it through antitoxin expression [(Blazejewski et al., 2019)](https://paperpile.com/c/Z6n5nF/BTkU). While these systems were shown to be able to prevent the escape of the majority of engineered organisms [(Chan et al., 2015)](https://paperpile.com/c/Z6n5nF/WCj5), or transfer of engineered DNA [(Blazejewski et al., 2019)](https://paperpile.com/c/Z6n5nF/BTkU) neither were 100% efficient. For example, very rare events such as mutations within the toxin gene component of the kill switches developed by Chan et al (2015) allowed a very small minority of individuals to break free from their control. Even a single escaped GMO would be unacceptable in a working anaerobic digester since it could rapidly divide into a much larger population [(Lenski, 1993)](https://paperpile.com/c/Z6n5nF/DTGa) (Figure 4). Biocontainment is therefore an important avenue for future research as an enabling technology for community monitoring and manipulation in AD.

# 7. Conclusions

Enhancing the tools available to monitor and manipulate AD microbially-driven processes could substantially improve control, yields and the utility of AD and other processes that rely on anaerobic microbial communities for function. Underlying many of the challenges associated with improving these tools is a need for a greater understanding of the mechanisms underpinning mixed microbial community interactions. Molecular methods are important for mechanistic discovery but too slow and expensive for routine or real-time diagnostics. Genetic manipulation tools demonstrated at laboratory scale require practical translation to industrial AD through robust process scale testing and consideration of GMO, ecological and biocontainment issues.

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**Author statement**

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**Figure Legends**

Figure 1: Schematic overview of a whole cell biosensor.

A stimulus (in this case a molecule: grey circles) enters the microbial biosensor cell (green oval) from the external environment and is recognised and bound by a regulatory protein (brown ovals). The stimulus / regulatory protein complex is now able to bind to its corresponding genetic regulatory region (curved red line) resulting in reporter gene induction (curved blue arrow) and subsequent reporter protein expression (blue circles). Accumulation of reporter protein results in an easily detectable phenotype of the biosensor cell (in this case blue fluorescence, depicted by blue dashed oval).

Figure 2: Comparison of visual and volatile-based reporter systems in the context of AD.

A microbial biosensor equipped with a visual reporter system (left) may have limited utility in an anaerobic digester since the visual signal would be difficult to detect within the non-transparent liquid phase of a digester. A microbial biosensor carrying a volatile-based reporter system (right) may be better suited for use in a digester since the volatiles (grey circles) produced by the enzyme the reporter gene codes for (pointed brown circles) reach the gas phase and can be sampled and detected non-invasively.

Figure 3: Niche creation within an AD community could promote invasion and persistence of an engineered organism.

A: An engineered microorganism (green oval) introduced into an anaerobic digester has to compete with endogenous microbial community members (red, blue, grey, pink, brown) for the niches they already occupy. As the engineered organism is likely to have impaired competitiveness, it is lost from the community (red cross). B: When introduced into an additional niche (such as a membrane-enclosed space: dashed black oval) the engineered organism is subjected to fewer competitive factors, potentially enabling it to successfully invade and persist.

Figure 4: Potential biocontainment risks.

A: Genetically modified microorganisms (green ovals) that provide a useful function mediated by an engineered gene (curved blue arrow) are introduced into an anaerobic digester. This population also possess a genetically encoded kill switch (blue switch symbol). Small molecule inducers (not shown) are added to the digester to suppress the kill switch, permitting cell survival. B: A small number of genetically engineered microorganisms escape the digester and enter the environment. Since this environment lacks the inducers required to prevent kill switch activation, the kill switch engages (closed switch symbols) in the majority of cases, resulting in cell death (red crosses). In a very small minority of cases, mutations within the kill switch prevent the toxin gene from being expressed in the absence of inducers (open switch symbol), allowing engineered microorganisms to persist in the environment (green oval without cross). C: No longer constrained by the kill switch, escapee microorganisms proliferate outside their intended environment.

Table1: Potential AD monitoring techniques summary.

(Amha et al., 2018; Chang et al., 2017; Cruz et al., 2021; De Vrieze et al., 2018; Lamb et al., 2019; Perpetuo et al., 2011; Renella, G., Giagnoni, L., 2016; Si et al., 2017; Sun et al., 2019; Vanwonterghem et al., 2014; Wu et al., 2019).

Table 2: Potential AD manipulation techniques summary.

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