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Article

Evaluation of Common Supermarket Products as Positive Controls in Biochemical Methane Potential (BMP) Tests

Konrad Koch [1,*], Sasha D. Hafner [2], Sergi Astals [3,4] and Sören Weinrich [5]

1. Chair of Urban Water Systems Engineering, Technical University of Munich, Am Coulombwall 3, 85748 Garching, Germany
2. Hafner Consulting LLC, Reston, VA 20191, USA; sasha@hafnerconsulting.com
3. Department of Chemical Engineering and Analytical Chemistry, University of Barcelona, C/Martí i Franquès 1, 08028 Barcelona, Spain; sastals@ub.edu
4. Advanced Water Management Centre, The University of Queensland, Brisbane, QLD 4072, Australia
5. Biochemical Conversion Department, Deutsches Biomasseforschungszentrum gemeinnützige GmbH, Torgauer Str. 116, 04347 Leipzig, Germany; soeren.weinrich@dbfz.de

* Correspondence: k.koch@tum.de; Tel.: +49-89-289-13706

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Abstract: Biochemical methane potential (BMP) tests are commonly applied to evaluate the recoverable amount of methane from a substrate. Standardized protocols require inclusion of a positive control with a known BMP to check the experimental setup and execution, as well as the performance of the inoculum. Only if the BMP of the positive control is within the expected range is the entire test validated. Besides ignorance of this requirement, limited availability of the standard positive control microcrystalline cellulose might be the main reason for neglecting a positive control. To address this limitation, eight widely available grocery store products have been tested as alternative positive controls (APC) to demonstrate their suitability. Among them, Tic Tacs and gummi bears were very promising, although they are dominated by easily degradable sugars and so do not test for hydrolytic performance. Coffee filters exhibited a similar performance to microcrystalline cellulose, while whole milk might be chosen when a more balanced carbohydrate:protein:lipid ratio is important. Overall, the approach of predicting the BMP of a substrate based on the nutritional composition provided on the product packaging worked surprisingly well: BMP of the eight tested products was 81–91% of theoretical maximum BMP based on nutritional information and generic chemical formulas for carbohydrates, proteins, and lipids.

Keywords: anaerobic digestion; biochemical methane potential test; positive control; microcrystalline cellulose; validation of test setup and inoculum performance

1. Introduction

Anaerobic digestion (AD) is used to stabilize organic material, while simultaneously recovering energy in the form of methane. Biochemical methane potential (BMP) tests are commonly used to determine the methane potential of a substrate [1–5]. In these tests, the substrate is mixed with an inoculum and methane production is measured over several days. Test conditions are meant to be optimal for the microbial community, to ensure maximum and relatively rapid degradation. This includes the selection of an appropriate inoculum-to-substrate ratio (ISR) and the addition of trace elements and vitamins in case the inoculum itself lacks them [2,6]. The inoculum is usually taken from an active and stable digester operated in a steady state, providing a highly diverse microbial community able to digest a large variety of organic substances [3]. Nevertheless, there are many reasons why an inoculum may exhibit insufficient performance, even if the same source is used frequently with satisfying results. These reasons include a temporary instability of the source digester, inadequate sampling (i.e., withdrawal from a badly mixed "dead zone") or storage, or a lack of adaptation to the substrate to be tested. In these cases, if the performance of the inoculum is not verified in a test, BMP may be underestimated.

The methane production of the inoculum is quantified by blanks (negative control; inoculum-only bottles). It comes from residual substrates, as well as from dead biomass from the microbial community itself [2–4]. Blanks are required to calculate net methane production from the substrate by subtracting inoculum production from the total gas production measured in a BMP test. The methane production by blanks, however, is not sufficient for verifying inoculum performance, because it provides no information on degradation of an external substrate [7,8]. Therefore, another set of assays should be reserved for a positive control, for which the BMP can be predicted. The positive control serves as an important quality check for both experimental setup and inoculum fitness, because its BMP should be within an expected range. For instance, a BMP higher than the theoretical maximum BMP of the positive control is a clear indicator of fatal flaws in the experimental setup or execution. Positive controls are often substrates that involve all steps of the AD process, i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis. For simplicity, these are usually pure substances with a known chemical composition. Besides tributyrin and oleic acid as a representative for lipids [3,9] and Bovin Serum Albumin as a representative for proteins [10], the one most commonly applied is microcrystalline cellulose (CEL) representing complex carbohydrates as the usually most dominant nutrient fraction in many organic substrates [3,4,11]. Its chemical formula of $(C_6H_{10}O_5)_n$ translates to a theoretical BMP of 414 L_{CH4}/kg [12]. However, this value is the theoretical maximum, which does not consider any use of substrate for production of microbial biomass.

The amount of the available substrate which is shunted to production of microbial biomass can hardly be generalized, since it depends on biomass yield, kinetics of biomass death and decay, and test duration [13]. Both calculated results based on measured biomass yields and measured BMP values show significant variability in cellulose BMP [14–16]. For instance, Heerenklage et al. [17] found that the amount of methane recovered from CEL was significantly lower for a lyophilized inoculum compared to a fresh one. They concluded that the microbial biomass yield was higher for the lyophilized inoculum due to the damaging conservation step. The operation of a BMP test in batch mode (only one single feeding event at the beginning) also contributes to a discrepancy in biomass yields observed in continuous operation. Owing to the lack of substrate towards the end of the test, the biomass formed will also decay and increase the percentage recovery of the positive control as methane with further incubation time [18]. A typical biomass yield for carbohydrates under anaerobic conditions (neglecting biomass decay) is around 15% [13,19]. According to recent guidelines, at least 81% [4,11] or 85% [3], and of course not more than 100%, of the theoretical value should be recovered. Even though defining the expected range for the BMP is a hard task considering these uncertainties, the performance in a test can also be evaluated empirical based on previously achieved values as long as the same substrate is used regularly. For instance, Dandikas et al. [20] used dried whole-crop maize, in addition to CEL, as a second internal positive control standard.

To underline the importance of the positive control, the VDI 4630 [4] strongly recommend its application, while for the VDLUFA Association Method [11], as well as for Holliger et al. [3], the integration of a positive control is a mandatory requirement for the validation of both test setup and performance of the inoculum. In case the required recovery as methane is out of the range mentioned, the test has to be rejected and repeated. The importance and potential utility of positive controls was demonstrated recently in an international inter-laboratory study [14]. By applying validation criterion, including limits on the positive control BMP, many extreme values could be excluded from the data set, reducing the standard deviation of the results for the shared substrates tested.

Despite its importance, the application of a positive control is unfortunately not uniformly used within the AD community. One reason might be lack of awareness of the recommendations of available guidelines and the lack of understanding that the validation criteria are not designed to create an extra effort, but instead, to help ensuring high-quality results. It has to be noted that in contrast to previous guidelines, the guideline of Holliger et al. [3] was published with open access to allow free access to the paper. Another reason for not including a positive control in a BMP test might be the fact that, particularly for labs located in the global south, the accessibility to purchase of high-quality chemicals from lab shops and the related costs are limiting factors, too. For instance, Donoso-Bravo et al. [21] reported that they used chopped cellulose-based paper as a positive control. It has been demonstrated that BMP tests can be conducted even with very simple apparatus while achieving a quality comparable to fully automated systems [22,23].

Owing to the importance of including a positive control in a BMP test to check both accuracy or faults in the experimental setup and the performance of the inoculum, alternative substrates suitable for use as positive controls, but more accessible and inexpensive, could be valuable for the biogas research community. The objective of the present work was to identify and compare such substrates to each other and to microcrystalline cellulose. Eight substrates were selected according to the following criteria:

- Ease of availability
- Affordability
- Consistent and homogeneous composition
- Ease of storage at room temperature and dose (no further pre-treatment needed)
- No inhibiting substances present

Furthermore, a suitable positive control should show the following behavior in a BMP test:

- Good digestibility (reasonably fast degradation)
- High reproducibility (i.e., low relative standard deviation (RSD) among replicates)
- Acceptable specific methane production (SMP) curve shape [24]

Ideally, the chemical composition of the positive control should be similar to the substrate(s) to be tested. This would facilitate checking the abundance of microorganisms responsible for all important degradation pathways, such as LCFA (long chain fatty acids) degraders as part of the degradation of lipids. Whether selecting CEL as a positive control for protein-rich or lipid-rich substrates is a suitable strategy has been a subject of debate [14]. Thus, depending on the substrate under investigation, alternative positive controls based on proteins or lipids should be evaluated as well. However, in comparison to CEL and typical substrate utilized for anaerobic digestion, mainly alternative positive controls with high shares of carbohydrates were investigated in the present study.

2. Materials and Methods

2.1. Characteristics of Alternative Positive Control Substrates

The substrates were chosen according to the criteria presented above. All products were purchased from a grocery store in Germany and used in their original form, except for coffee filters, which were cut into 1 cm strips with scissors. The characteristics of all substrates can be found in Table 1. Chemical composition was provided by the supplier as nutritional information on the packaging of each product, while total solids (TS) and volatile solids (VS) were determined in the laboratory according to standard methods [25]. Based on nutritional information, total carbohydrates are composed of sugars, fibers and other carbohydrates (mainly starch). Thus, mass of other carbohydrates was calculated by subtracting fiber and sugar mass from reported total carbohydrate mass. Nutritional information was also used to calculate VS (as sum of carbohydrates, proteins, and fats), which was compared to measured values. A photograph of the packaging for each substrate can be found in the Supplementary Material (Table S1).

Table 1. Characteristics of microcrystalline cellulose and of substrates chosen as an alternative positive control. Price has been calculated based on the current supermarket price in Germany. Macromolecular composition according to supplier, VS was measured in the laboratory and calculated from nutritional information.

Name	Price (€/kg_{VS})	Chemical Composition (g/kg_{FM})					Ch:Pr:Li Ratio [b] (-)	VS (g_{VS}/kg_{FM})	
		Carb. [a]	Sugar	Fiber	Protein	Lipid		Calc.	Meas.
Cellulose	101.0	1000	0	0	0	0	1:0:0	1000	1000
Coffee filters	13.5	1000 [c]	0 [c]	0 [c]	0 [c]	0 [c]	1:0:0	1000	916
Cornflakes	8.8	760	80	30	70	9	97:8:1	949	905
Gummi bears	5.7	310	460	0	69	5	154:14:1	844	859
MPP [d]	8.2	596	54	87	89	53	14:2:1	879	853
Oat flakes	2.9	548	12	110	140	67	10:2:1	877	868
Tic Tacs	28.7	30	945	0	1	5	975:1:5	981	990
Wheat flour	1.6	713	7	0	100	10	72:10:1	830	858
Whole milk	11.5	0	47	0	34	35	1.3:1:1	116	113

[a] Other carbohydrates (reported total carbohydrate mass minus sugar and fiber mass). [b] Carbohydrate: Protein: Lipid ratio (carbohydrates include sugar and fiber). [c] Assumed to be 100% cellulose (coffee filters include no nutritional information on packaging). [d] Mashed potato powder.

The price per kg of VS ranges clearly between 1.6 € for wheat flour to 28.7 € for Tic Tacs, but still remains much below the price for microcrystalline cellulose of about 100 €/kg_{VS}. The VS content calculated from nutritional information align quite well with the VS content determined in the lab with a discrepancy <5% of fresh mass for all substrates (excluding coffee filters, which, as a non-food product, do not come with nutritional information). Although results cannot be generalized to all batches and manufacturers of these products, this consistency provides some indication that label nutrition information is quite accurate.

The substrates chosen cover a broad range in chemical composition. Coffee filters are composed of the fiber form of cellulose and could be considered as an easily available alternative to microcrystalline cellulose. Most of the other substrates are dominated by carbohydrates, although their complexity differs. Gummi bears and Tic Tacs are mainly composed of sugars, flour mainly of starch, while corn flakes, mashed potato powder, and oat flakes contain starch but also a share of dietary fiber, according to the supplier's information (Table 1) with an assumed lower degradability or at least slower degradation rate. Being composed of mainly simply sugars, Tic Tacs will likely show a faster degradation than other substrates, but also tend to acidification if the inoculum lacks enough alkalinity. Furthermore, its degradation will not check the hydrolysis step. Whole milk differs from all other substrates by having a low VS concentration and a nearly balanced carbohydrate:protein:lipid ratio.

In order to evaluate the BMP achieved, the theoretical maximum BMP of the substrates (assuming 100% degradability and no substrate partitioning to cell synthesis) was estimated based on the nutrient composition given by the supplier and presented in Table 1 by applying the theoretical methane potentials for individual nutrients based on their chemical composition, as presented by Boyle [12] (calculations were double-checked by the *Online Biogas App* (OBA) [26]):

- 414 L_{CH4}/kg_{VS} for complex carbohydrates (starch, cellulose, fiber) assuming model substrate cellulose/starch ($C_6H_{10}O_5$) [6,27],
- 392 L_{CH4}/kg_{VS} for simple carbohydrates (sugars) assuming model substrate sucrose ($C_{12}H_{22}O_{11}$) [28,29],
- 534 L_{CH4}/kg_{VS} for proteins assuming empirical formula $C_4H_{6.1}O_{1.2}N$ [27,30], and
- 1006 L_{CH4}/kg_{VS} for lipids assuming model substrate tripalmitin ($C_{51}H_{98}O_6$) [27,31].

2.2. Source and Characteristics of Inoculum

Digested sewage sludge used as inoculum was collected from Garching wastewater treatment plant (Bavaria, Germany) and was used immediately without any pre-treatment or degassing. This plant mainly treats municipal wastewater from approximately 45,000 population equivalents. The anaerobic digester is fed with a mixture of primary and secondary sludge at an organic loading rate of about 1.0 $kg_{VS}/(m^3 \cdot d)$. The digester is operated at mesophilic conditions (approximately 38 °C) with a hydraulic retention time of about 30 days. The inoculum was characterized by a TS concentration of 26.5 ± 0.3 g_{TS}/ kg_{FM} (mean ± standard deviation, $n = 3$) and a VS/TS ratio of 64.6 ± 1.4%.

2.3. Biochemical Methane Potential (BMP) Tests

BMP tests were conducted using the Automatic Methane Potential Test System II (AMPTS II; Bioprocess Control, Sweden) following the guideline of Holliger et al. [3,32]. Due to limited capacity, and in order to avoid any bias by the inoculum used in each experiment, the substrates were tested in single assays in three independent trials. Blanks (inoculum-only bottles), as well as CEL, were carried out in triplicate in each trial. The ISR was 2 on a VS basis [3,4] resulting in a fresh matter mass of 30 g for whole milk and about 4 g for the other substrates. Operating volume was 450 mL and a mixing mode of 5 min mixing and 25 min rest was used. All tests were carried out under mesophilic conditions (38 ± 1 °C) at least until net daily methane production was less than 1% of the cumulative net gas production for three consecutive days [3], which is indicated by diamonds in the following graphs. Prior to incubation, all bottles were flushed with a mixture of N_2 and CO_2 (65% N_2, 35% CO_2) at a flow rate of 2.5 L per minute for 30 s to ensure a more than 5-times statistical exchange of the entire headspace of about 200 mL [33]. The AMPTS II system reports standardized (dry, 0 °C, 1 atm) cumulative methane volume from each bottle, which can be used to calculate both specific methane production (SMP) curve and final BMP. BMP was calculated following the standard approach [34] and double-checked by the *Online Biogas App* (OBA) [26].

3. Results and Discussion

The application of a positive control is an important prerequisite for the validation of both experimental test setup and inoculum performance. However, access to the commonly applied CEL might be limited in certain regions of the world due to availability or affordability. Furthermore, by the application of CEL as a positive control, only the performance of carbohydrate degraders are tested, while other potentially similar important pathways (i.e., for protein and lipid degradation) are not checked/tested at all. The results presented below document the suitability of particular alternative positive controls (APCs), but also support the general idea of using grocery store goods as APCs, whose use is doubtless better than no positive control at all.

3.1. Specific Methane Production and Specific Methane Production Rate

The average specific methane production of CEL ($n = 9$, triplicate in three trials) and the eight alternative positive control substrates ($n = 3$, single assay in three trials) tested are depicted in Figure 1. All specific methane production (SMP) curves show the typical behavior with a steep initial increase followed by a stable plateau phase, with only minor methane production [24]. Only CEL and coffee filters exhibited a minor lag-phase (below 1 day). Except for whole milk, all specific methane production (SMP) curves are similar to CEL, presumably because they are all dominated by carbohydrates. Lipids make up 28% of the volatile solids in whole milk, causing a significantly higher methane yield compared to carbohydrates and proteins [6,27]. Diamonds indicate the time when the daily gas production was below 1% of net cumulative production for three consecutive days, being the criterion for test termination [3]. A suitable alternative positive control (APC) should reach the termination criterion in a reasonable duration, while checking the fitness of the intended degradation pathways, such as hydrolysis. Tic Tacs, wheat flour and gummi bears reached the criterion slightly earlier than CEL, whereas oat flakes, whole milk, mashed potato powder, coffee filters, and cornflakes reached the criterion slightly later than CEL (see Table 2). For a closer look at what happened in the initial phase, Figure 2 presents the average specific methane production rate during the first 5 days of incubation.

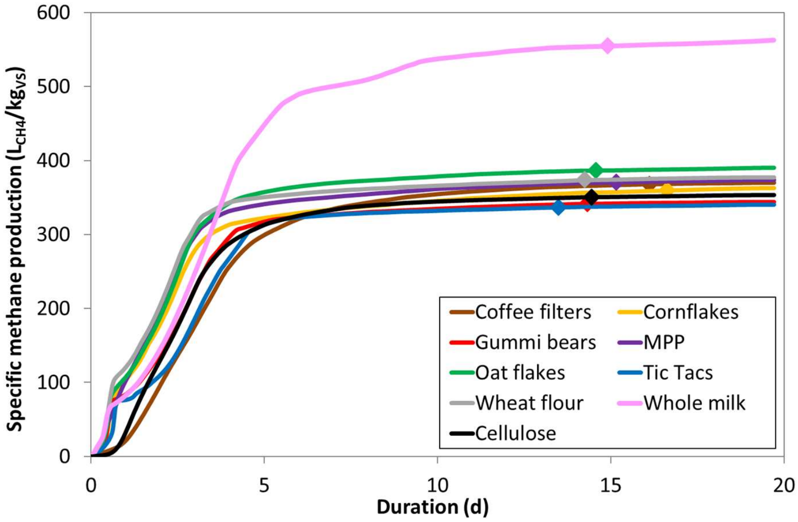

Figure 1. Average specific methane production of microcrystalline cellulose (n = 9) and the eight alternative positive control substrates (n = 3) tested. Diamonds indicate the time when the daily gas production was below 1% of cumulative production during three consecutive days.

Table 2. Summary of time to reach $BMP_{1\%\ net,\ 3d}$ as well as a comparison of theoretical maximum and measured BMP (average and standard deviation) for cellulose and the alternative positive controls tested.

Name	Time to Reach $BMP_{1\%\ net,\ 3d}$ (d)	Theoretical Max. BMP [a] (L_{CH4}/kg_{VS})	Measured BMP (L_{CH4}/kg_{VS})	$BMP_{meas.}/BMP_{theo.}$ (%)
Cellulose	14.5 ± 1.2	414	353 ± 12.4	85.3
Coffee filters	16.1 ± 1.4	414	370 ± 9.2	89.3
Cornflakes	16.7 ± 0.6	427	363 ± 5.6	85.1
Gummi bears	14.4 ± 0.7	415	344 ± 2.1	82.9
MPP	15.2 ± 1.0	460	374 ± 11.8	81.1
Oat flakes	14.6 ± 0.7	478	390 ± 9.2	81.6
Tic Tacs	13.5 ± 0.5	396	340 ± 1.2	85.9
Wheat flour	14.2 ± 0.9	435	378 ± 8.5	86.7
Whole milk	15.0 ± 0.5	619	563 ± 12.9	90.9

[a] Theoretical maximum BMP from elemental composition (Section 2.1).

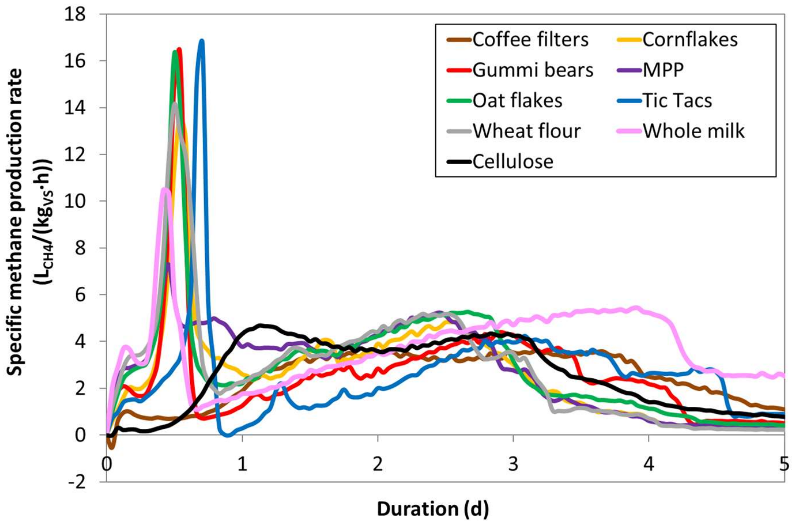

Figure 2. Average specific methane production rate of microcrystalline cellulose (n = 9) and the eight alternative positive control substrates (n = 3) tested in the first 5 days of incubation.

CEL exhibited the typical short lag-phase of about half a day, followed by a broad peak of moderate methane production before flattening out after 4 days of incubation. Coffee filters showed similar behavior, but the rate was only gently inclining causing a time delay compared to CEL. While the powder-form of CEL provides a high surface, the coffee filters were only cut into strips. A more intensive grinding of the coffee filters would probably contribute to further converge the two curves. To what extent the difference in degradation kinetics is also caused by the form the cellulose was provided, i.e., microcrystalline vs. fibrous, remains open. Still, the intention of a positive control is to check whether the expected amount/range of methane is recovered in an experimental setup, while the kinetics of the degradation process are actually less important.

Gummi bears, oat flakes, and wheat flour, and to a lesser extent whole milk and mashed potato powder (MPP), showed a peak methane production after about half a day reaching specific methane production rates of more than 16 $L_{CH4}/(kg_{VS}·d)$, while leveling off around 2 to 6 $L_{CH4}/(kg_{VS}·d)$ for the next three to four days. Interestingly, Tic Tacs showed the highest peak, which occurred about four hours later than for gummi bears. Since Tic Tacs are composed only of sugar, this delay is surprising at first glance. Assuming that the ISR of 2 was properly chosen to avoid an accumulation of volatile fatty acids (VFA), the reason for the minimal delay in reaching the methane production peak compared to gummi bears or even more complex substrates, such as wheat flour and oat flakes, was probably that—according to the packaging—carnauba wax was applied as a glazing agent, which potentially delayed the dissolution of sugar.

3.2. Difference in Specific Methane Production Relative to Cellulose

Microcrystalline cellulose is considered the standard positive control for BMP tests [2–4]. In order to better visualize differences in the SMP curve, Figure 3 displays the absolute difference in the specific methane production between each alternative positive control and CEL. In accordance with Figure 1, whole milk achieves a BMP about 200 L_{CH4}/kg_{VS} higher than cellulose due to its relatively high content of proteins and particularly lipids (Table 1). Whole milk has two potential disadvantages. First, it has a very high water content (88%), contributing to a further dilution of the assay, which can eventually lead to an inhibition of the AD process [35,36]. Second, whole milk is among those substrates that reaches the plateau phase relatively late, as can be also seen from the continuous positive slope of the curve even towards the end of the experiment. One potential criterion for the selection of a suitable positive control is probably its fast biodegradability, in order to limit the necessary duration of the test to a minimum. Last but not least, whole milk has potentially the highest variability among manufacturers and even between batches from the same manufacturer and its conservation is also quite limited compared to the other APCs, which can be stored without chilling for several months. Milk powder might be a superior APC to fresh milk due to its higher TS concentration, homogeneity, and longer storability. However, it was not tested in the present study.

Wheat flour, oat flakes, MPP, and cornflakes depict a very similar behavior of two initial peaks during the lag-phase of CEL followed by convergence reaching finally a surplus between 10 and 37 L_{CH4}/kg_{VS}. The curves for gummi bears and Tic Tacs are characterized by only one initial peak followed by a similar (gummi bears) or even lower (Tic Tacs) methane production compared to CEL achieving a minimal negative value (−9 and −13 L_{CH4}/kg_{VS}, respectively). Among all substrates tested, gummi bears had the most CEL-like methane production. Still, owing to its high share of easily degradable sugar, the buffer capacity of the inoculum is challenged more than in the case of CEL.

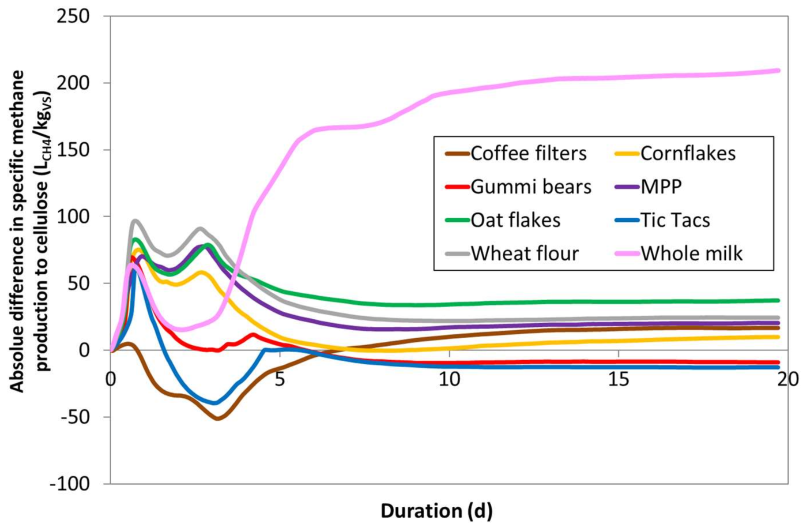

Figure 3. Absolute difference in the specific methane production relative to microcrystalline cellulose of the eight alternative positive control substrates tested.

3.3. Biochemical Methane Potential (BMP)

The role of a positive control is to validate both test setup and performance of inoculum by generating an amount of methane that lies within the expected range of its theoretical maximum BMP, considering that some of the substrate will be used for producing microbial biomass [13]. A well-suited positive control is characterized by a BMP with high reproducibility (i.e., low standard deviation among replicates) that should preferably be reached within a short digestion time and check at least those degradation pathways required by the substrate(s) to be tested. The latter, however, is often not done when using MCC as a positive control. When comparing the time to reach the defined termination criterion $BMP_{1\%\text{ net, 3d}}$ [3] in Figure 4, one has to keep in mind that not all substrates requires hydrolysis to be performed. There is potentially no need to check the hydrolytic performance of the selected inoculum for substrates rich in readily available organic matter, such as food waste. The shortest test duration is required for Tic Tacs being composed of sugar only, while most of the other substrates are in a similar range as CEL. Corn flakes is the only APC that needs significantly longer than CEL, although the difference is only two days.

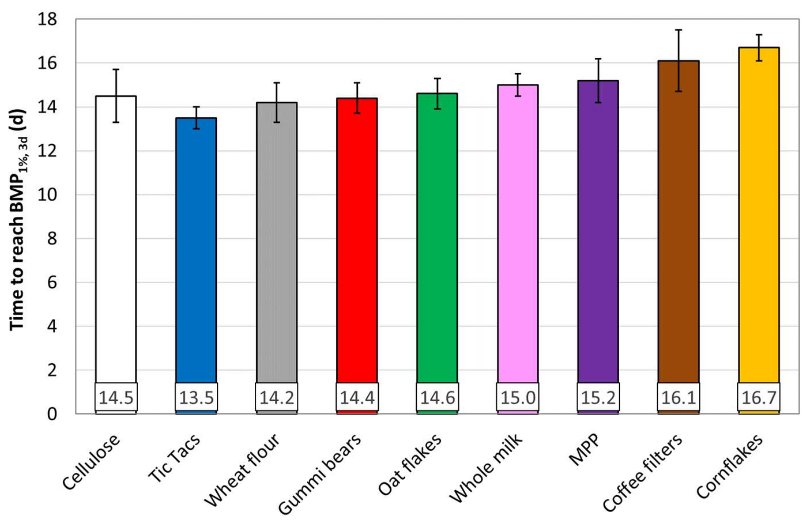

Figure 4. Time to reach the termination criterion $BMP_{1\%\text{ net, 3d}}$ for microcrystalline cellulose and alternative positive control substrates. Error bars indicate standard deviation ($n = 9$ for microcrystalline cellulose and $n = 3$ for all other substrates). Values have been sorted in ascending order.

BMP of CEL and the alternative positive control substrates tested is presented in Figure 5. BMP for CEL ranged from 337 to 377 L_{CH4}/kg_{VS} among 9 replicates, with an average of 353 L_{CH4}/kg_{VS} (see also Table 2). A total of 5 out of 9 replicated did not meet the validation criteria proposed by Holliger et al. [3] of >352 L_{CH4}/kg_{VS}, while they all matched the minimum of 335 L_{CH4}/kg_{VS} recommended by VDI 4630 [4] and VDLUFA [11]. Potentially, this observation calls for a reconsideration of the defined minimum of Holliger et al. [3]. Except for whole milk, all APCs had a lower standard deviation than CEL. With a relative standard deviation of 0.4% and 0.6%, respectively, Tic Tacs and gummi bears had by far the lowest values, and hence the highest precision among the substrates tested. The BMP of CEL and coffee filters are not significantly different, which implies that the assumption that coffee filters are composed of cellulose seems to be reasonable.

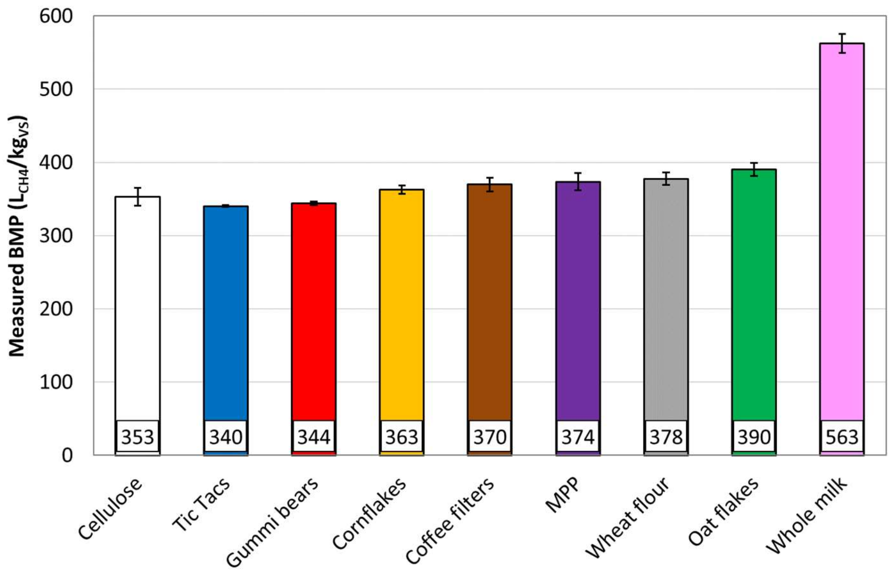

Figure 5. Measured BMP for microcrystalline cellulose and alternative positive control substrates. Error bars indicate standard deviation (n = 9 for microcrystalline cellulose and n = 3 for all other substrates). Values have been sorted in ascending order.

3.4. Overall Assessment

An ideal positive control is characterized by a high and fast anaerobic degradability (i.e., considerable methane production and reasonable time to reach the termination criterion) and a high reproducibility (i.e., low standard deviation among the replicates), while ideally checking all degradation pathways the substrate(s) to be tested require. As most guidelines recommend CEL as a positive control independent from the substrate to be tested [2–4,11], future attempts to improve existing guidelines should take this into account. Table 2 summarizes the most important findings. The time to reach $BMP_{1\%\ net,\ 3d}$ was relatively similar for all substrates tested and ranged between 13.5 and 16.7 days. On average, 353 L_{CH4}/kg_{VS} was recovered from CEL, which corresponds to 85% of the theoretical value. Coffee filters even recover 89% of their theoretical value, indicating a slower but higher biodegradability of the fiber compared to the microcrystalline form. All APCs tested achieved between 81% (MPP, oat flakes) and 91% (whole milk) of their theoretical maximum BMP. That whole milk achieved the highest recovery was expected, because the other candidates are all dominated by carbohydrates whose degradation has a higher partitioning for synthesis of microbial biomass [18]. Products with dietary fibers (i.e., corn flakes, MPP, and oat flakes) achieved a lower recovery, implying that their digestibility is lower compared to other complex carbohydrates, such as starch in wheat flour.

Whole milk has a high share of proteins and lipids, which are known to have lower microbial biomass yields than carbohydrates [18]. Hence, over 90% of the theoretical value was recovered during the BMP test. This is actually a good argument for choosing a positive control with less carbohydrate, since the uncertainty for the acceptable range of the BMP is narrower. With a relative standard deviation (RSD) of 3.5%, the variation of the BMP for CEL was below the recommended limit of 5% [3]. Surprisingly, the RSD of all APCs except for whole milk were even lower, with a minimum of 0.4% for Tic Tacs and 0.6% for gummi bears.

Tic Tacs had the lowest RSD in the measured BMP; they reached the termination criterion the fastest, and also the standard deviation among the replicates of this time was the lowest. A potential downside of Tic Tacs is the fact that it is composed of easily degradable sugar only, potentially leading to souring (acidification) of the process in case the inoculum is not well-buffered, neither requiring hydrolysis nor the presence of other microorganism groups besides sugar degraders. Gummi bears can at least compensate this disadvantage somehow, containing also starch, some proteins and little lipids as well, while achieving otherwise a similar good performance. It is worth mentioning that distinguishing between complex carbohydrates (including both starch and fiber, represented by the chemical formula of $C_6H_{10}O_5$; max. theoretical BMP of 414 L_{CH4}/kg_{VS}) and simple sugars (represented by the chemical formula of $C_{12}H_{22}O_{11}$; max. theoretical BMP of 392 L_{CH4}/kg_{VS}) is important to guarantee a good approximation of the theoretical maximum BMP. This differentiation is fortunately often provided on the packaging, as it is also important from a dietary point of view. While glucose is often used as model substrate for sugars [27,37], it is assumed that sucrose is the most dominant form of sugars in groceries [38]. Whole milk might be chosen when a balanced ratio between (simple) carbohydrates, proteins and lipids is needed in order to test the performance of all kinds of degraders, although hydrolysis is not required/checked either. Coffee filters are potentially a good alternative when CEL is not available.

3.5. General Recommendations

The grocery store products selected demonstrated their applicability as alternative positive controls. They were selected according to the list of properties presented and also their behavior in the BMP test was generally convincing. Whether all products are available in a similar high quality also in other parts in the world is hard to judge. The results of this study clearly illustrated that widely available products can be used as alternative positive controls in case of limited access of CEL. Some of them are potentially even superior to CEL, as they cover a broader range of degradation pathways for their digestion. It is even possible to create a mixture of some of the products to obtain a positive control of a desired composition. Combining whole milk and coffee filters, for instance, could provide a positive control with a balanced carbohydrate:protein:lipid ratio, and a mixture of simple and complex carbohydrates, including fiber. Consistent composition and quality (especially for biological products such as whole milk) should be guaranteed at all time.

There are an endless number of grocery products around the globe, and the ones chosen for this study were somewhat arbitrary. However, the main conclusion of this study is that the general idea of using common grocery store goods as positive controls, while calculating the theoretical maximum BMP from the nutritional information on the packaging, is a simple but reasonable approach. Only determination of the VS concentration in the laboratory is strongly recommended in order to verify the package information. Considering the uncertainties related to degradability of the substrate, and also to the substrate partitioning to cell synthesis, a recovery of at least 80% of maximum theoretical BMP should be achieved for all substrates tested. For substrates with a more balanced carbohydrate:protein:lipid ratio, such as whole milk, a recovery of around 90% should be achieved.

As long as there is no high quality product or mixture that has demonstrated its suitability in a comprehensive international interlaboratory test, CEL will likely remain the most commonly applied positive control, although the findings of this study imply that there are some products that are even superior to CEL because of their high reproducibility, good degradability, and kinetics, as well as the fact that all three degradation pathways can be evaluated. Still, if CEL is not available as a positive control in a BMP test, using an alternative positive control as proposed is always better than no positive control at all.

4. Conclusions

Measurement of the BMP of a positive control is a critical prerequisite for BMP test validation. Limited access to microcrystalline cellulose might be a reason for not always including a positive control. A selection of alternative positive controls were tested in this study. All substrates were easily available, affordable and easily degradable under anaerobic conditions. Some substrates include hydrolysis as a required step, some do not. Most substrates were dominated by carbohydrates, while others also contained proteins or lipids, allowing us to also check the performance of other degraders. The specific methane production curves showed relatively similar behavior for all substrates tested, except for some deviation, especially for easily degradable sugars. At least 80% of the theoretical maximum BMP was achieved within reasonable digestion times.

A good positive control is characterized by reasonably fast degradation and a high reproducibility among the replicates. All substrates tested were suitable for use as a positive control, and the general approach of trusting nutritional info by suppliers on substrate's package for estimating the maximum BMP seems valid. Tic Tacs and gummi bears are promising alternative positive controls. However, they do not provide a check on the hydrolytic capacity of the inoculum, nor on degradation of proteins and lipids. Whole milk or oat flakes might be selected when a more balanced ratio between carbohydrates, proteins and lipids is desired, while coffee filters are an alternative to microcrystalline cellulose, exhibiting relatively similar behavior. In order to check the performance of the main pathways required for the test substrate to be degraded, the positive control's composition should be as similar as possible to the test substrate. Blending several positive controls can potentially help to approximate the substrate's composition.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/5/1223/s1, Table S1: Photographs of the packages from the substrates chosen as alternative positive controls.

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