RESEARCH ARTICLE SUMMARY

MICROBIAL ENGINEERING

A heterogeneous microbial consortium producing short-chain fatty acids from lignocellulose

Robert L. Shahab, Simone Brethauer, Matthew P. Davey, Alison G. Smith, Silvia Vignolini, Jeremy S. Luterbacher, Michael H. Studer*

INTRODUCTION: Consortia of microorganisms are a promising alternative to monocultures for complex biotransformations because of their inherent advantages, such as the distribution of the metabolic burden by division of labor, the ability to convert complex substrates more efficiently, and their modularity. Consortia are also inherently more stable and less susceptible to contamination than monocultures. However, achieving stable and reproducible ratios of the different strains and their performance is challenging, especially for microbial consortia where mutual beneficial interactions are missing—for example, if one strain secretes cellulolytic enzymes to provide carbohydrates as a public good. Moreover, the need to find matching fermentation conditions in well-mixed reactors limits the selection of possible consortium members and prevents the harnessing of the full power of artificial consortia.

RATIONALE: To tackle these challenges and to advance the understanding and engineering of synthetic microbial consortia, we attempted to mimic one of the well-known natural

mechanisms for consortium stabilization: the spatial organization, along several different gradients, of abiotic factors within a biofilm. To this end, we established a membrane-aerated biofilm reactor, which allows for the creation of an oxygen-replete niche in an otherwise anoxic environment, to harbor artificial cross-kingdom microbial consortia for the consolidated bioprocessing of lignocellulose to short-chain fatty acids (SCFAs). Because many SCFA producers have a limited carbohydrate substrate spectrum, the heterogeneous lignocellulosic sugar mixture is funneled to lactate as the central intermediate.

RESULTS: The lactate platform consists of the aerobic fungus *Trichoderma reesei*, which secretes cellulolytic enzymes and thus provides soluble sugars; facultative anaerobic lactic acid bacteria (LAB); and the obligate anaerobic lactate-fermenting strain, which provides the product. To set up the consortia and to form the necessary oxygen gradient that allows the growth of all members, we applied a sequential inoculation scheme starting with the fungus, followed by LAB, and lastly the lactate fer-

Principle of the lactate platform. A membrane-aerated bioreactor featuring an oxygen gradient (depicted at the bottom) allows for the consolidated bioprocessing of lignocellulose to short-chain fatty acids by a heterogeneous consortium consisting of an aerobic, cellulolytic fungus growing as a biofilm in the oxygen-replete spatial niche, a facultative anaerobic lactic acid bacterium to funnel the carbohydrate mixture to lactate, and a lactate-consuming obligate anaerobic bacterium for product formation.

menter. We found that the metabolic activity of *T. reesei* alone is not sufficient to create an environment with a redox potential suitable for obligate anaerobes; rather, it requires the additional presence of LAB. We tested the feasibility of the lactate platform to produce butyric acid using *Clostridium tyrobutyricum*, which co-consumes lactic acid and acetic acid in a 3:1 molar ratio, thereby valorizing this often unused component of lignocellulose and allowing for a higher carbon yield relative to direct carbohydrate conversion. We were able to produce a stable cocultivation of the three strains, and, with a mixture of cellulose and xylose as model substrate, we obtained 0.35 g of butyric acid per gram of carbohydrates—a value slightly exceeding yields achieved with soluble substrates or by addition of commercial cellulolytic enzymes. With beechwood as substrate, a yield equivalent to 196 kg of butyric acid per metric ton of feedstock was reached. Switching to *Veillonella criceti*, an obligate anaerobic bacterium unable to utilize carbohydrates, a mixture of propionic and acetic acid was produced from lignocellulose, underpinning the suitability of the lactate platform approach to compensate for missing metabolic capabilities of product-forming strains. Longer SCFAs such as valeric and caproic acid, which are more valuable than the C₂ to C₄ SCFAs, could be produced by integrating *Megasphaera elsdenii*, a strict anaerobic rumen bacterium able to perform chain elongation, into the lactate platform. By extending this configuration with *V. criceti*, the ratio of odd- and even-numbered SCFAs could be tuned, and the valeric acid concentration was higher than with the three-member community by a factor of nearly 3. To demonstrate the platform's wide applicability, we identified several other chemicals—including alcohols (ethanol, propanol, butanol, 1,2-propanediol), polyhydroxybutyrate, and lipids—that could be produced through the lactate platform.

CONCLUSION: The lactate platform demonstrates several inherent benefits of synthetic consortia, such as their modularity and their ability to convert complex mixed substrates into valuable platform chemicals. The use of spatial niches in a membrane reactor—not limited to oxygen, but also for other abiotic factors such as temperature, light, or pH—expands the available toolbox for successfully engineering stable and controllable synthetic communities for numerous novel applications. ■

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Cite this article as R. L. Shahab et al., *Science* 369, eabb1214 (2020). DOI: 10.1126/science.abb1214

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https://doi.org/10.1126/science.abb1214

RESEARCH ARTICLE

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A heterogeneous microbial consortium producing short-chain fatty acids from lignocellulose

Robert L. Shahab^{1,2}, Simone Brethauer², Matthew P. Davey³, Alison G. Smith³, Silvia Vignolini⁴, Jeremy S. Luterbacher¹, Michael H. Studer^{2*}

Microbial consortia are a promising alternative to monocultures of genetically modified microorganisms for complex biotransformations. We developed a versatile consortium-based strategy for the direct conversion of lignocellulose to short-chain fatty acids, which included the funneling of the lignocellulosic carbohydrates to lactate as a central intermediate in engineered food chains. A spatial niche enabled in situ cellulolytic enzyme production by an aerobic fungus next to facultative anaerobic lactic acid bacteria and the product-forming anaerobes. *Clostridium tyrobutyricum*, *Veillonella criceti*, or *Megasphaera elsdenii* were integrated into the lactate platform to produce 196 kilograms of butyric acid per metric ton of beechwood. The lactate platform demonstrates the benefits of mixed cultures, such as their modularity and their ability to convert complex substrates into valuable biochemicals.

Lignocellulosic biomass is an abundant source of fixed, renewable carbon and is a promising alternative to fossil petroleum as feedstock for a range of chemicals. Short-chain fatty acids (SCFAs) with two to six carbon atoms are currently produced from fossil resources and have been suggested as potential bio-derived platform molecules (1). However, the combination of the heterogeneous carbohydrate composition of lignocellulose and the limited metabolic flexibility of many SCFA producers leads to low product yields and undesirable by-product formation (fig. S1A). A more homogeneous carbon source for SCFA producers can be provided by metabolically funneling the heterogeneous mixture of carbohydrates in the biomass to a central platform molecule in a primary fermentation, followed by a secondary conversion to the desired product (fig. S1B). An ideal system would combine the primary and secondary fermentation, as well as the depolymerization of the carbohydrates by cellulolytic enzymes, in a single processing step termed consolidated bioprocessing (CBP) (2).

The engineering of such complex reaction cascades into a single microbial host can create a substantial metabolic burden that limits the overall productivity of the system (3). Consequently, low carbon fluxes remain a major challenge for CBP approaches based on genet-

ically engineered microorganisms (4, 5), which are often characterized by metabolic imbalances (6–8). This limitation could be overcome by a metabolic division of labor (3), which is also observed in natural consortia, where complex reaction cascades are common and biochemically difficult tasks are catalyzed by multiple species according to the principle of metabolic compartmentalization (9). Each member of the community occupies its ecological niche and is specialized in a subtask or exchanges different beneficial molecules such as vitamins or amino acids. However, the high complexity of natural consortia makes the targeted engineering of the desired trait difficult and limits their industrial application for the production of biochemicals. Alternatively, less complex and more defined synthetic consortia can be engineered (10) and have been applied for CBP of lignocellulose to produce isobutanol (11), ethanol (12, 13), or acetone, butanol, and ethanol (14). A major challenge in the design of such bottom-up consortia is the requirement of overlapping tolerance ranges of several abiotic process parameters such as oxygen concentration, pH, and temperature, which limits the selection of possible consortium members (15). Thus, the majority of coculture studies reported to date were carried out using only two species (10) with similar abiotic requirements, and few examples of artificial consortia with several populations have been reported (16–19).

Furthermore, under homogeneous growth conditions, population control and stable reproducible performance are challenging to achieve, especially for microbial consortia where mutual beneficial interactions are missing (9, 20, 21)—for example, if one strain secretes cellulolytic enzymes to provide carbohydrates as a public good (22). In such cooperator/cheater communities, different growth rates of consortium

members may lead to overgrowth of one population and extinction of the other (23). To tackle these challenges, we proposed to switch from a homogeneous to a heterogeneous reactor system that provides defined spatial niches where different abiotic conditions fitted to each consortium member (e.g., oxygen, temperature, light) prevail in the otherwise homogeneous reactor environment (24). This enforced niche partitioning can stabilize microbial communities of, for example, an aerobic and facultative anaerobes (24–27) and enables the cocultivation of microorganisms with non-overlapping abiotic requirements.

Here, we demonstrate the feasibility of engineering spatial and metabolic niches as a strategy to build and control artificial cross-kingdom microbial consortia and introduce a CBP strategy termed the “lactate platform” where lactic acid is used as a central and versatile intermediate for the direct production of different SCFAs from lignocellulose.

Many SCFA producers are noncellulolytic strains of bacteria with limited flexibility to utilize different carbohydrates, but with the ability to metabolize lactic acid. This ability makes lactic acid a potential central intermediate in a microbial consortium-based CBP strategy aiming at the conversion of the complete carbohydrate fraction of lignocellulose to the target product (fig. S1B). The lactate platform consists of (i) the aerobic fungus *Trichoderma reesei* for the cooperative secretion of cellulolytic enzymes, which provide soluble sugars; (ii) facultative anaerobic lactic acid bacteria (LAB) that are able to convert all types of solubilized sugars to lactic acid; and (iii) the lactate-fermenting anaerobic product-forming strain (Fig. 1A). In contrast to other abiotic factors such as temperature or pH (Fig. 1B), the oxygen requirements of the members of the artificial consortium do not overlap (Fig. 1C). By using a membrane-aerated reactor in combination with the facultative anaerobic LAB, an oxygen gradient is generated in the reactor that allows the redox potential requirements of all strains to be fulfilled (Fig. 1D). To build the consortia, we followed a three-step inoculation scheme: First, the reactor was inoculated with *T. reesei* to allow the formation of a fungal biofilm on top of the membrane (Fig. 1E and fig. S2). After 48 hours, the LAB was inoculated (designated as phase I with the time set to $t = 0$ hours), followed by the subsequent inoculation of the lactate-fermenting strain(s) after waiting another 48 hours (beginning of phase II). All insoluble substrates were added to the reactor at the time of the fungal inoculation, and soluble substrates were fed to the reactor after inoculation with LAB.

Butyric acid production

To produce butyric acid, we set up a synthetic consortium consisting of *T. reesei*, *Lactobacillus*

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(A) Selected metabolic pathways in the community-based CBP using lactate and acetate as central intermediates. The table (upper right) shows the capability of strains to metabolize polymers (cellulose and hemicellulose), oligomers (gluco- and xylo-oligosaccharides), C₅/C₆ sugars, lactate (LA), and acetate (AA). (B) Approximate tolerance ranges for pH and temperature of strains (42). (C) Requirements of strains for dissolved oxygen concentration. (D) Schematic representation of the membrane-aerated bioreactor for CBP of lignocellulosic biomass to SCFAs with dissolved oxygen concentration profile. (E) Photograph of mature fungal biofilm on the membrane.

Fig. 2. CBP of cellulose and xylose to butyric acid.

(A) In the base case, 17.5 g liter⁻¹ Avicel and 9.32 g liter⁻¹ xylose (fed over a time period of 75 hours; dashed blue line) were converted to butyric acid by a consortium of *T. reesei*, *L. pentosus*, and *C. tyrobutyricum* at an oxygen transfer rate (OTR) of 3.24 hour⁻¹. **(B to D)** Differences from the base case: **(B)** Acetic acid feed to 2 g liter⁻¹ over a time period of 75 hours (dashed red line), **(C)** addition of *L. brevis* to the consortium, **(D)** reduction of OTR to 0.34 hour⁻¹. Stages are indicated at the top; stage III depicts the condition in which *C. tyrobutyricum* lacks acetic or lactic acid. **(E)** Ternary plot of theoretical butyric acid yield for feedstocks containing different fractions of glucan, xylan, and acetic acid (see supplementary materials). **(F)** Two-stage fermentation of the yield-optimized glucose/acetate mixture. In stage I, glucose was fermented by *L. pentosus* to lactic acid, followed by acetic acid addition and inoculation with *C. tyrobutyricum* in phase II. **(G)** Observed and theoretical butyric acid concentration and yield for the lactate platform. Data in (A) to (D) are means $\pm$ SD of two independent experiments; data in (F) and (G) are means $\pm$ SD of four independent experiments. The box in (G) denotes SD of the mean; error bars denote the 99% confidence interval.

pentosus (for lactic acid production from glucose and xylose), and *Clostridium tyrobutyricum* (for producing the target product). *C. tyrobutyricum* is able to convert glucose, xylose, or a mixture of lactic and acetic acid to butyric acid (Fig. 1A and table S1). We first used a mixture of cellulose (17.5 g liter⁻¹) and xylose (9.32 g liter⁻¹), which represented a model substrate for lignocellulose (Fig. 2A). The metabolic activity of *T. reesei* reduced the dissolved oxygen content below the detection limit within 8 to 12 hours after fungal inoculation and lowered the redox potential to values

between +20 and -20 mV (fig. S3), which is not suitable for obligate anaerobes. Consequently, the cocultivation of *T. reesei* and the obligate anaerobe *C. tyrobutyricum* was not possible (fig. S4). Upon inoculation of *L. pentosus*, the redox potential dropped to less than -300 mV by reduction of oxygen to either hydrogen peroxide or water for the regeneration of nicotinamide adenine dinucleotide (NAD⁺) by NADH oxidases (table S1) (28), thereby creating suitable growth conditions for *C. tyrobutyricum*. Butyric acid production began after 100 hours and was accompanied by a decrease

of lactic and acetic acid. A maximum concentration of 4.1 g liter⁻¹ (0.14 g g⁻¹ fermentable sugars, 93.1% selectivity, 0.02 g total SCFA liter⁻¹ hour⁻¹) was reached at the end of phase II (table S2). At the beginning of phase III, lactic acid utilization by *C. tyrobutyricum* stopped, presumably because lactic acid cannot serve as a sole carbon source but must be co-consumed with either glucose or acetic acid (fig. S5, A to E) (29). To verify this hypothesis, we fed acetic acid (2 g liter⁻¹) as an additional carbon source. Indeed, this enabled the complete utilization of lactic acid to produce a butyric

Fig. 3. CBP of cellulose and xylose to propionic and acetic acid or mixed SCFAs. (A) CBP of 35 g liter⁻¹ Avicel and 18.64 g liter⁻¹ xylose (fed over a time period of 150 hours) to propionic and acetic acid with *V. criceti* as the lactate-fermenting strain. **(B)** CBP of 17.5 g liter⁻¹ Avicel and 9.32 g liter⁻¹ xylose (fed over a time period of 75 hours) to mixed SCFAs with *M. elsdenii* as the lactate-fermenting strain. Stage III depicts the condition in which *V. criceti* or *M. elsdenii* lack lactic acid. Data are means $\pm$ SD of two independent experiments.

acid concentration of 10.7 g liter⁻¹ (0.37 g g⁻¹, 77.6% selectivity, 0.05 g total SCFAs liter⁻¹ hour⁻¹; Fig. 2B and table S2).

As an alternative to the addition of acetic acid, we aimed to enhance the metabolic flux from carbohydrates to acetic acid by extending the microbial consortium with the obligate heterofermentative strain *L. brevis*, which produces lactic and acetic acid from both hexoses and pentoses via the phosphoketolase (PK) pathway (table S1). In comparison with the base-case consortium (Fig. 2A), the extended microbial consortium produced a higher amount of acetic acid in phases I and II (Fig. 2C). This enabled the complete reutilization of lactic acid, leading to a butyric acid concentration of 9.5 g liter⁻¹ (0.33 g g⁻¹, 77.9% selectivity, 0.06 g total SCFAs liter⁻¹ hour⁻¹; table S2). The lower butyric acid selectivity relative to the base case is due to the slight overproduction of acetic acid.

Finally, we tested elevating the acetic acid concentration in the system by minimizing the previously reported degradation by *T. reesei* (26), which could be limited by the amount of oxygen entering the reactor. Indeed, decreasing the oxygen transfer rate by a factor of ~ 10 by using a siloxane membrane with a thicker wall led to a higher concentration of accumulated acetic acid in phase II (Fig. 2D). Then, lactic acid could be completely converted to butyric acid at a concentration of 10.2 g liter⁻¹ after 372 hours (0.35 g g⁻¹, 96.7% selectivity, 0.03 g total SCFAs liter⁻¹ hour⁻¹; table S2).

To obtain a benchmark for conventional butyric acid production, we also performed simultaneous saccharification and fermentation experiments with the same substrates, using only *C. tyrobutyricum* and a cellulolytic enzyme cocktail. A yield of 0.34 g g⁻¹ butyric acid

with a selectivity of 82% (fig. S7) was achieved, which is slightly lower than the corresponding results of the lactate platform (table S2).

Influence of substrate composition on theoretical butyric acid yields

To evaluate and compare the different options for producing butyric acid from lignocellulosic biomass and model substrates, we calculated the theoretical yields as a function of the substrate composition and the chosen fermentation route. To determine the theoretical yields of the lactate platform, we assumed that all hexoses are fermented by *L. pentosus* through the Embden-Meyerhof-Parnas pathway to lactic acid, although *C. tyrobutyricum* is also able to consume glucose. This assumption is justified, because *L. pentosus*—in contrast to *C. tyrobutyricum*—can use cellobiose intracellularly, which favors the metabolic flux of hexoses to lactic acid (Fig. 1A) (30). Furthermore, the cessation of lactic acid consumption by *C. tyrobutyricum* upon acetic acid depletion observed in the experiments described above (Fig. 2A) is a strong indication that no glucose is available for this strain, as lactic acid can also be metabolized in the presence of glucose (fig. S5, F to J). We also assume that xylose is metabolized through the PK pathway to lactic acid and acetic acid solely by *L. pentosus* (table S1). These products, as well as the acetic acid present in the feedstock, are then converted by *C. tyrobutyricum* to butyric acid. We experimentally determined the required ratio of lactic to acetic acid and found that 3 mol of lactic acid and 1 mol of acetic acid were converted to 2 mol of butyric acid (fig. S5, A to E), which is in line with literature data (29). In fig. S6, we propose the biochemical basis of the lactate/acetate-

cofermenting pathway to butyric acid and compare it to the glucose fermentation to butyric acid in *C. tyrobutyricum*.

The yield based on the mass of fermentable carbohydrates for direct sugar conversion varies only slightly between 0.486 g g⁻¹ for glucose and 0.493 g g⁻¹ for xylose; however, it ranges from 0 g g⁻¹ (for pure glucan) up to 0.635 g g⁻¹ (81% glucan, 19% acetic acid) for the lactate platform (Fig. 2E). Because acetic acid is typically an unused resource in real biomass, only the carbohydrates were counted as carbon source. If acetic acid is counted as part of the substrate, the maximal theoretical butyric acid yield of the lactate platform is 0.52 g g⁻¹ carbon source, which is 7% higher than the theoretical yield of the direct sugar conversion. For a feedstock composition corresponding to that of pretreated beechwood (68.5% glucan, 24.3% xylan, 7.2% acetic acid), the theoretical yield of butyric acid produced via the lactate platform is 0.525 g g⁻¹ fermentable carbohydrates compared to 0.488 g g⁻¹ for the direct fermentation of the carbohydrates. To underpin this theoretical framework with experimental data, we determined the butyric acid yield of the lactate platform using the optimal mixture of glucose and acetic acid. To this end, glucose was first metabolized to lactic acid using *L. pentosus*, followed by the addition of *C. tyrobutyricum* and acetic acid in the second phase of the fermentation. A butyric acid yield of 0.53 g g⁻¹ glucose or 0.43 g g⁻¹ carbon source, respectively, could be achieved, which corresponds to 109.4 $\pm$ 2.7% of the theoretical yield for direct glucose conversion by *C. tyrobutyricum* (Fig. 2, F and G). This result also outperforms published yields for fermentations of the same substrates using natural or genetically engineered strains (table S4).

Propionic and acetic acid production

To demonstrate the modularity of the lactate platform, we replaced *C. tyrobutyricum* with *Veillonella criceti* to produce propionic acid. *V. criceti* is an obligate anaerobic bacterium that is unable to utilize carbohydrates but converts lactic acid to propionic and acetic acid (Fig. 1A and table S3). Thus, the lactate platform approach compensates for the missing metabolic flexibility of the strain and enables the production of propionic acid from carbohydrates. CBP of 17.5 g liter⁻¹ microcrystalline cellulose and 9.32 g liter⁻¹ xylose yielded 8.9 g liter⁻¹ total carboxylic acids (fig. S8 and table S5). When doubling both carbon sources, 13.7 g liter⁻¹ acetic acid and 9.3 g liter⁻¹ propionic acid were produced (Fig. 3A and table S5).

Mixed C₂ to C₆ SCFA production

Higher SCFAs such as valeric (C₅) and caproic (C₆) acid are more valuable than the lower SCFAs (C₂ to C₄). However, few microbial species are known to produce these compounds in

Fig. 4. Steam pretreatment of beechwood and CBP to SCFAs. (A) Mass fluxes of glucan, xylan, acetic acid, and lignin in the two-stage steam pretreatment (further details in fig. S9). (B) Experimental yields of CBP with different microbial consortia to various SCFAs (fig. S10).

substantial amounts (31). One of the exceptions is *Megasphaera elsdenii*, a strict anaerobic rumen bacterium. *M. elsdenii* favors lactic acid over glucose as carbon source and is not able to metabolize xylose or cellulose (32, 33), thereby making it a suitable candidate for the lactate platform (Fig. 1A). We tested the cocultivation of *T. reesei*, *L. pentosus*, and *M. elsdenii* in the membrane-aerated reactor following the subsequent inoculation strategy described above. With 17.5 g liter⁻¹ cellulose and 9.32 g liter⁻¹ xylose as substrates, we recorded the simultaneous accumulation of 4.3 g liter⁻¹ acetic acid, 1.2 g liter⁻¹ propionic acid, 5.0 g liter⁻¹ butyric acid, 1.5 g liter⁻¹ valeric acid, and 1.3 g liter⁻¹ caproic acid in 280 hours. The obtained SCFA yields were 0.46 ± 0.01 g SCFA per g fermentable sugars (Fig. 3B and table S6), showing the successful cocultivation of the three strains. However, after inoculation of *M. elsdenii* the redox potential increased (~1.5 mV hour⁻¹ from *t* = 50 hours to *t* = 200 hours), indicating that the metabolic activity of *T. reesei* and *L. pentosus* was not sufficient to maintain a sufficiently low redox potential in longer fermentation runs, likely due to product toxicity.

CBP of pretreated beechwood

After successfully converting model substrates, we targeted the direct conversion of a lignocellulosic substrate—pretreated beechwood—to the different target products. We applied a two-stage steam pretreatment procedure to recover temperature-sensitive solubilized xylo-oligomers and acetic acid in a moderately inhibitory liquid stream termed prehydrolyzate after a mild first pretreatment and a well-digestible solid substrate after the second, more

severe pretreatment step (Fig. 4 and fig. S9). For CBP of beechwood, we added 3% (w/w) pretreated solids at the beginning of the fermentation and started the first-stage prehydrolyzate feed with the inoculation of *L. pentosus*. With *C. tyrobutyricum* as lactic acid-fermenting strain, the consortium produced 9.5 g liter⁻¹ butyric acid (0.38 g g⁻¹ fermentable carbohydrates, 91.5% selectivity, 0.014 g total SCFA liter⁻¹ hour⁻¹; fig. S10A and table S2), which corresponds to an overall yield of 196.5 kg of butyric acid per metric ton of dry raw beechwood (Fig. 4B). This yield was approximately twice as high as the reported yields of a natural consortium (34), an anaerobic coculture (35), or a natural single cellulolytic strain (36) and was even comparable to yields achieved on lignocellulosic hydrolysates (37, 38), although we did not add external enzymes (table S4). When *V. criceti* was used as fermenting strain in the lactate platform, 6.7 g liter⁻¹ acetic acid and 5.6 g liter⁻¹ propionic acid (0.49 g g⁻¹ fermentable carbohydrates, 0.031 g total SCFA liter⁻¹ hour⁻¹; fig. S10B and table S5) were produced. This corresponded to 133.3 kg of acetic acid and 113.6 kg of propionic acid per metric ton of raw biomass (Fig. 4B). Finally, when *M. elsdenii* was integrated into the lactate platform, a mixture of 6.8 g liter⁻¹ acetic acid, 4.2 g liter⁻¹ butyric acid, 2.4 g liter⁻¹ caproic acid, and small amounts of propionic and valeric acid (each ~1 g liter⁻¹) (fig. S10C and table S6) could be produced from pretreated beechwood. Overall, the process yielded 0.42 g of total SCFAs per gram of total fermentable sugars, corresponding to 217.1 kg of total SCFAs per metric ton of raw beechwood with a productivity of 0.012 g liter⁻¹ hour⁻¹. Even more than 600 hours after inoculation of *M. elsdenii*, the

accumulation of metabolic end products of the anaerobic pathways such as caproic acid was recorded, indicating that the reduced oxygen transfer rate achieved by using a thicker membrane prolonged the metabolic activity of the strict anaerobic strain (fig. S10C and table S6). This is in line with the measured smaller slope of the redox potential (~0.23 mV hour⁻¹ from *t* = 400 hours to *t* = 1094 hours) in comparison to the approach with the thinner membrane (Fig. 3B).

Finally, we aimed to influence the ratio of odd- or even-numbered SCFAs produced by *M. elsdenii* (Fig. 1A). *M. elsdenii* can take up extracellular propionic acid, which is activated intracellularly to propionyl-coenzyme A (CoA) by a propionyl-CoA transferase (PCT) (Fig. 1A). Because PCT has a broad substrate spectrum, the functional group CoA can be swapped between acetyl-CoA and SCFA (39, 40), which is essential for the chain elongation process. On this basis, we hypothesized that an increased availability of propionic acid increases the production of higher, odd-numbered SCFAs. Because *V. criceti* was shown to convert lactic acid to propionic and acetic acid, thereby increasing the concentration of propionic acid, we set up a lactate platform containing both *V. criceti* and *M. elsdenii* as lactate-fermenting strains. Using pretreated beechwood as substrate, this artificial consortium produced 2.6 g liter⁻¹ acetic acid, 0.8 g liter⁻¹ propionic acid, 6.7 g liter⁻¹ butyric acid, 3.5 g liter⁻¹ valeric acid, and 2.1 g liter⁻¹ caproic acid (Fig. 4B and fig. S10E). Thus, the valeric acid concentration was higher by a factor of nearly 3 relative to the mixture produced by the community without *V. criceti*. The process yield was 0.42 g of total SCFAs per gram of total fermentable

sugars and 0.22 g of total SCFAs per gram of raw beechwood, similar to the community without *V. criceti*.

The metabolism of *M. elsdenii* also allows the reverse β -oxidation of butyric acid (electron acceptor) taken from the environment to caproic acid if an electron donor such as lactate is available (Fig. 1A). Thus, by extending the community with *C. tyrobutyricum* instead of *V. criceti*, we sought to increase the share of even-numbered longer SCFAs. By doing so, we measured a SCFA mixture consisting of 4.6 g liter⁻¹ acetic acid, 1.0 g liter⁻¹ propionic acid, 7.4 g liter⁻¹ butyric acid, 1.4 g liter⁻¹ valeric acid, and 2.5 g liter⁻¹ caproic acid (Fig. 4B and fig. S10D). Relative to the base case, the butyric acid concentration increased by a factor of 1.75 and the acetic acid concentration decreased by a factor of 0.66. However, the presence of *C. tyrobutyricum* did not increase the caproic acid concentration, which indicates the lack of electron donors that are required for chain elongation by *M. elsdenii* (41). Overall, the yield achieved with the community extended with *C. tyrobutyricum* increased to 0.46 g of total SCFAs per gram of total fermentable sugars and 0.24 g of SCFAs per gram of raw beechwood.

CBP of lignocellulosic substrates to mixed SCFAs can also be performed by natural mixed cultures such as the rumen microbiome or different kinds of anaerobic digestion consortia, where methane formation is inhibited. These processes are summarized as the “carboxylate platform” (1) and are typically performed with undefined waste substrates, making a comparison of the yields with those of the lactate platform difficult. However, the yield that we achieved with the lactate platform is comparable to reported *M. elsdenii* fermentations on glucose or corn stover hydrolysate, and we even exceeded the reported titers (table S4). Furthermore, in the lactate platform, xylose was also converted, which is not possible in axenic *M. elsdenii* cultures.

In the batches run on beechwood, we also quantified hydrolytic enzyme expression over time to determine whether *T. reesei* was metabolically active and was producing enzymes during all phases of the fermentations. We consistently found increasing activities of cellobiohydrolases, endoglucanases, β -glucosidases, and xylanases in the supernatants after inoculation with the lactic acid and product-forming strains (fig. S11). Together with further experiments described in figs. S18 to S20, we conclude that *T. reesei* was metabolically active throughout the experiments.

Discussion and outlook

In this study, we merged synthetic microbial ecology with process engineering to realize and control artificial food chains distributed over as many as four highly diverse micro-

organisms. Our goal was to provide a platform that minimizes the required metabolic capabilities of product-forming microorganisms to facilitate their integration into an artificial community utilizing all biomass fractions. To demonstrate the platform's wide applicability, we identified several other bulk or fine chemicals, including acetic acid, alcohols (ethanol, propanol, butanol, 1,2-propanediol), polyhydroxybutyrate, and lipids, that could be produced through the lactate platform (fig. S12). We analyzed the stoichiometries of the required pathways and found that, relative to the alternative direct sugar fermentation routes for acetic acid, polyhydroxybutyrate, ethanol, and butanol, the lactate platform theoretically boosts the carbon flux from lignocellulose to the target product (fig. S12 and tables S7 to S9). A switch to another target product may require the exchange of the lactate-fermenting strain(s), and as a consequence, different abiotic requirements such as pH, temperature, or presence of co-substrates will likely have to be fulfilled. Furthermore, for some products the artificial food chain must be extended; that is, consortia containing at least four different microorganisms would be necessary. Thus, the creation of additional spatial and/or metabolic niches is desirable to fully harness the potential of the lactate platform. To this end, we propose several possibilities to adapt the biofilm membrane reactor (24). For instance, temperature gradients could be created by installing local heat sinks or sources in the reactor, or pH gradients could be formed by introducing carbon dioxide or ammonia through a membrane to the reactor (fig. S13). In the same way, co-substrates such as hydrogen or carbon monoxide could be fed to the consortium. Furthermore, light management in the reactor could facilitate the mixotrophic growth of microalgae on lignocellulosic feedstocks, which paves the way to a range of valuable products such as lipids for biodiesel production or omega-3 fatty acids as aquatic feed. As a first step in the direction of the latter, we showed that *T. reesei* and the microalgae *Chlamydomonas reinhardtii* were able to grow together in an illuminated aerobic biofilm using pure cellulose as a substrate (figs. S14 to S17). Prior to a potential industrial application, further optimization of the lactate platform is necessary; in particular, the product concentrations and the corresponding productivities need to be enhanced. At this proof-of-concept stage, the obtained productivities in the range of 0.01 to 0.06 g liter⁻¹ hour⁻¹ (tables S2, S5, and S6) compare well with literature data (table S4). One reason for the moderate productivities is the gap between the optimal cellulase production temperature (28° to 30°C) and the optimal temperature for enzyme activity (40° to 50°C). Besides using an advanced industrial cellulase-producing strain, the use of a temperature gradient reactor could also

increase the productivity. The bulk phase could be operated at elevated temperatures while the enzyme-producing biofilm could be cooled via the membrane.

Taken together, the results achieved with the lactate platform demonstrate the inherent benefits of mixed artificial cultures, such as their modularity and their ability to convert complex substrates to valuable platform chemicals. This reactor engineering complements the available toolbox for successfully engineering stable and controllable synthetic communities for numerous novel applications.

Materials and methods

Fungal, bacterial, and algal strains and culturing methods

L. pentosus (DSM-20314), *L. brevis* (DSM-20054), *C. tyrobutyricum* (DSM-2637), *V. criceti* (DSM-20734), and *M. elsdenii* (DSM-20460) were purchased from Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures (Germany).

One ml of glycerol stock was inoculated to MRS medium for precultures of *Lactobacillus* in 500-ml Erlenmeyer flasks and incubated for 48 hours at 150 rpm at 33°C. The MRS medium contained (g liter⁻¹): peptone from casein, 10; meat extract, 10; yeast extract, 5; glucose, 20; Tween 80, 1; K₂HPO₄, 2; sodium acetate, 5; ammonium citrate, 2; and MgSO₄·7H₂O, 0.2. The pH was adjusted to 6.2–6.5 with hydrochloric acid.

C. tyrobutyricum and *M. elsdenii* precultures were cultivated in a reinforced clostridial medium (RCM). Composition of the RCM medium (in g liter⁻¹): yeast extract, 13; peptone, 10; glucose, 5; soluble starch, 1; sodium chloride, 5; sodium acetate, 3; L-cysteine-HCl, 0.5; agar, 0.5.

V. criceti precultures were cultivated in medium containing (in g liter⁻¹): trypticase (BBL), 5; yeast extract, 3.0; Na-(DL)-lactate, 7.5; Na-thioglycolate, 0.75; Tween 80, 1.0; glucose, 1.0; Na-resazurin solution [0.1% (w/v)], 0.5 ml. The medium for *C. tyrobutyricum* and *V. criceti* was boiled under continuous flushing with nitrogen and the pH was adjusted to 6.8 before it was aliquoted to 100-ml serum bottles (50 ml in each) and autoclaved at 121°C for 20 min.

V. criceti is known for its high nutrient requirements (43, 44), but we could show that it is still active on Mandels medium containing only 1.0 g liter⁻¹ complex media components. We found that the fermentation time was prolonged by a factor of ~3 without further supplementation of complex nutrients, while the yields remained the same (table S3). Therefore, we used Mandel medium in the CBP experiments with *V. criceti*.

All serum bottles were inoculated from a 2-day liquid culture stored at 4°C and incubated on an orbital shaker at 140 rpm at 37°C.

T. reesei Rut-C30 (ATCC 56765) was purchased from the VTT, Finland. Spores from a

7-day-old potato dextrose agar slant culture were incubated at 28°C and stored at 4°C. For precultures, Mandels medium containing 7.5 g liter⁻¹ microcrystalline cellulose (Avicel PH-101, Sigma Aldrich) was inoculated with spores from the agar slant in an Erlenmeyer flask for 4 days at 28°C and 150 rpm. Mandels medium contained the following ingredients (g liter⁻¹): KH₂PO₄, 2; (NH₄)₂SO₄, 1.4; MgSO₄·7H₂O, 0.3; CaCl₂·6H₂O, 0.4; urea, 0.3; peptone, 0.75; yeast extract, 0.25; and trace element stock, 1 ml liter⁻¹. The trace element stock contained (g liter⁻¹): FeSO₄·7H₂O, 5; MnSO₄·H₂O, 1.6; ZnSO₄·7H₂O, 1.4; CoCl₂·6H₂O, 3.7; and 10 ml liter⁻¹ concentrated hydrochloric acid, and was sterile filtered. To avoid precipitation, 100× CaCl₂ and MgSO₄ solutions were autoclaved separately before merging with the remaining ingredients.

Stocks of *C. reinhardtii* wild-type strain (WT12) were stored at room temperature in TAP medium. TAP medium contained per liter: TRIS, 2.42 g; TAP salt stock, 25 ml; phosphate solution, 0.375 ml; six Kropat's trace element stock solutions, 1 ml each; and glacial acetic acid, 1 ml. The medium was autoclaved for 20 min at 121°C. TAP salt stock solution contained (in g liter⁻¹): NH₄Cl, 15.0; MgSO₄·7H₂O, 4.0; CaCl₂·2H₂O, 2.0. The phosphate solution contained 28.8 g K₂HPO₄ and 14.4 g KH₂PO₄ and water was added to a volume of 100 ml. Kropat's trace element stock solutions contained: (1) 25 mM EDTA-Na₂; (2) 28.5 mM (NH₄)₆Mo₇O₂₄; (3) 2 mM CuCl₂·2H₂O and 2 mM EDTA; (4) 2.5 mM ZnSO₄·7H₂O and 2.75 mM EDTA; (5) 6 mM MnCl₂·4H₂O and 6 mM EDTA; (6) 20 mM FeCl₃·6H₂O, 22 mM EDTA, and 22 mM Na₂CO₃. For liquid precultures of *C. reinhardtii*, Erlenmeyer flasks were filled with fresh TAP medium and inoculated with 5% (v/v) stock solution and incubated at 25°C with an 18 hours light to 6 hours dark cycle with 120 mol m⁻² s⁻¹ for 5 days under shaking at 120 rpm.

Membrane bioreactor

The membrane bioreactors were based on Multifors 2 and Labfors 5 bioreactors (Infors HT, Switzerland) with 0.5 and 2.7 liter working volume, respectively. Both reactors were modified with a tubular polydimethylsiloxane (PDMS) membrane. The membrane area to volume ratio was 0.3 cm² ml⁻¹ at 0.5 liter scale. Mono-Lumen Tubing 0.64 × 1.19 × 0.28 mm (Dow Corning) gave a k_La value with standard error of 3.24 ± 0.05 hour⁻¹ in the Multifors reactors. The membrane area to volume ratio was 0.3 cm² ml⁻¹ in the Labfors. Mono-Lumen Tubing 1.58 × 3.18 × 0.80 mm (Dow Corning) membrane gave a k_La value with standard error of 0.34 ± 0.01 hour⁻¹. The membrane was continuously flushed with air at 140 ml min⁻¹ per liter liquid phase. The temperature was set to 30°C and the pH to 6.0 using 4 N phosphoric acid and 4 M sodium hydroxide.

The redox potential and the pH were measured using EasyFerm Plus ORP Arc and EasyFerm Plus PHI Arc probes (Hamilton, Switzerland). Liquid samples were collected using a UV lamp as the contamination barrier. No external cellulolytic enzyme mixture or nitrogen purging were used. The reactor was autoclaved for 20 min at 121°C with microcrystalline cellulose or pretreated beechwood. The remaining medium components were added when the reactor was inoculated with 5% (v/v) *T. reesei*. After 48 hours, the lactic acid bacteria were inoculated to an optical density (OD₆₀₀) of 0.5 by centrifuging the cells at 3000 rpm for 10 min and suspending them in fresh Mandels medium. This time was set as time zero. Another 48 hours after inoculation of the LAB, the *C. tyrobutyricum* or *V. criceti* was inoculated to 5% (v/v) from a 2-day-old pre-culture. For fed-batch experiments, the feed solution was sterile filtered and fed at a constant feeding rate.

Experiments with the green alga *C. reinhardtii* (WT12) were performed in a custom made 1-liter glass stirred tank reactor equipped with the same membrane as in the Labfors fermenters described above and a membrane area to volume ratio of 0.3 cm² ml⁻¹. In order to enable mixotrophic growth of the microalgae, a radially emitting light fiber (side glow fiber, 1.5 mm diameter, Starscape, UK) connected to an external light source (Cree XLAMP XHP 70.2 – 3000 K 80 CRI) was mounted next to the membrane (figs. S14 and S15). The reactor was autoclaved for 20 min at 121°C with water and microcrystalline cellulose. The remaining medium components were added when the reactor was inoculated at the same time with 5% (v/v) *T. reesei* and 5% (v/v) *C. reinhardtii*. For coculture experiments, Mandel's medium was used and the temperature was set to 26°C.

Steam pretreatment and beechwood analysis

Beechwood chips (*Fagus sylvatica*) from a local forest in Bern, Switzerland, were air-dried (dry matter 94%) and milled to <1.5 mm. The raw biomass was composed of [% (w/w)] glucan 40.0 ± 1.3, xylan 19.0 ± 0.5, acetic acid 7.3 ± 0.2, acid-insoluble lignin 25.6 ± 0.7, acid-soluble lignin 5.3 ± 0.1, ash 0.5, and extractives 0.9. A two-stage steam pretreatment was applied with a custom-built steam gun (Industrieanlagen Planungsgesellschaft m.b.H., Austria) to target the partial recovery of soluble xylo-oligosaccharides from hemicellulose in the prehydrolyzate and simultaneously maximize the glucose yield in the enzymatic hydrolysis of the solids. Beechwood was heated to 180°C by the injection of saturated steam and pretreated for a residence time of 24.8 min (severity of 3.75). At constant pressure and temperature, the liquid phase was removed through a circular nozzle located slightly above the lower

ball valve. Subsequently, the pressure was slowly released to 2.0 bar, which is below the known pressure needed to see an effect of the explosion (45). At 2.0 bar, the lower ball valve opened and the solids were removed from the reactor. The solids were composed of [in % (w/w)] 49.0 ± 0.6 glucan, 7.6 ± 0.1 xylan, 4.2 ± 0.1 acetic acid, 29.9 ± 0.7 acid-insoluble lignin, and 3.7 ± 0.4 acid-soluble lignin. The recovered solids were treated a second time at 230°C for 14.1 min and the pressure was abruptly released. The solids contained [in % (w/w)] 55.0 ± 1.4 glucan, 2.1 ± 0.1 xylan, 1.2 ± 0.2 acetic acid, 35.4 ± 0.8 acid-insoluble lignin, and 2.5 ± 0.1 acid-soluble lignin. The 180°C liquid fraction contained (in g liter⁻¹): acetic acid, 2.1; formic acid, 0.4; xylose, 1.1; xylo-oligosaccharides calculated as xylan, 22.4. During acid hydrolysis, an additional 5.57 g liter⁻¹ acetic acid was released from deacetylation of xylo-oligomers. The severity was calculated according to Overend *et al.* (46). Mass fluxes of glucan, xylan, acetic acid, and lignin are shown in Fig. 4A; see fig. S9 for further details of recovery percentages.

Enzyme assays

EG, BG, CBH, and xylanase activity assays were performed at pH 5.0 in the presence of 50 mM citrate buffer, 0.02 (w/v) % sodium azide at 50°C in duplicates. The enzymatic activity was expressed in international units (IU). One unit is defined as the amount of enzyme that catalyzes the conversion of one micromole of substrate per minute. EG, BG, and CBH assays were performed as described (47). Ultralow-viscosity carboxymethylcellulose [2 (w/w) %] was used as substrate for EG assays, Avicel [2 (w/w) %] for CBH, and 4-nitrophenyl α-D-glucopyranoside (1 mM) for BG. Birch wood xylan [1 (w/w) %] was used as substrate for xylanase activity measurements (12.5 μl sample in 125 μl total reaction volume). Reducing sugars were quantified using the 3,5-dinitrosalicylic acid (DNS) method (48).

Analytical methods

Acetic acid, lactic acid, propionic acid, butyric acid, formic acid, glucose, cellobiose, xylose, hydroxymethylfurfural, and furfural were quantified by high-performance liquid chromatography (Waters 2695 Separation Module, Waters Corp., Milford, MA) using an Aminex HPX-87H column (Bio-Rad) at 65°C with 5 mM H₂SO₄ as the mobile phase, flow at 0.6 ml min⁻¹, a refractive index detector (Waters 410) at 40°C, and a photodiode array detector (Waters 2998) at 210 nm. The detection limit was 0.05 g liter⁻¹. Structural carbohydrates and lignin were measured according to standard methods (49).

Chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids in biofilms of *T. reesei* and *C. reinhardtii* were quantified by first extracting pigments with dimethylformamide (DMF) and measuring the absorbance at 480,

647, and 664.5 nm (Thermo Spectronic UV-1, Thermo Fisher). Prior to the measurement, samples with a specific volume or mass were centrifuged in a 1.5-ml Eppendorf vial, 1 ml DMF was added to the pellet, and the vials were agitated using a vortex at room temperature for 15 min. The vials were centrifuged at 10,000 rpm on a benchtop centrifuge. The chlorophyll a, chlorophyll b, and total chlorophyll contents were calculated using the equations from (50) and the amount of carotenoids using the equation from (51).

Autofluorescence from photosynthetic pigments of the stained biofilm samples was also measured using a confocal laser scanning microscope (Leica SP8, Leica Microsystems). The setup was equipped with a white light laser set to 30% laser power to excite the sample. The scan speed was 400 Hz and the objective HC PL APO CS2 40×/1.30 oil with a magnification of 40 was installed. Autofluorescence from photosynthetic pigments was detected in the red channel (excitation at 650 nm and emission at 680 to 700 nm). Fluorescein diacetate was used as viability stain. Fluorescein was detected in the green channel (excitation 495 nm and emission at 514 to 521 nm). The fluorescence projections of the biofilm were generated using Leica LAS software.

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ACKNOWLEDGMENTS

We thank Genecor-DuPont for providing the enzymes used in this work and S. G. Oliver for generously providing us with *S. cerevisiae* FYdelta pet191::KanMX4. **Funding:** Supported by the Swiss National Science Foundation in the framework of National Research Program NRP 70 “Energy Turnaround” grant 407040-153868 (M.H.S.); Biotechnology and Biological Sciences Research Council (BBSRC) Innovate UK Industrial Biotechnology Catalyst

grant BB/M018210/01 “Developing Platforms for the Production of Diterpenoids” (A.G.S.); BBSRC Global Challenges Research Fund grant BB/S0014020/1 “Enhancing cobalamin (vitamin B12) bioavailability in culturally appropriate foods in India” (A.G.S.); and Leverhulme Trust grant RPG-2017-077 “Snow Algae—are they the most abundant photosynthetic organisms in terrestrial Antarctica?” (A.G.S.). The study has been conducted in the framework of the SCCER BIOSWEET, supported by the Swiss Confederation through Innosuisse–Swiss Innovation Agency (M.H.S.).

Author contributions: R.L.S., S.B., J.S.L., A.G.S., S.V., and M.H.S.

conceived the study and designed the experiments; R.L.S. conducted the experiments; and R.L.S., S.B., M.P.D., J.S.L., A.G.S., S.V., and M.H.S. analyzed the experimental results; R.L.S. and S.B. drafted the manuscript. All authors critically revised the article and approved the final manuscript. **Competing interests:** R.L.S. and M.H.S. submitted an international patent application (EP 2019/061402) on a method for the microbial production of fatty acids and lipids from lignocellulose with a consortium of at least three microorganisms.

Data and materials availability: All data are available in the main text or the supplementary materials.

SUPPLEMENTARY MATERIALS

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14 February 2020; accepted 26 June 2020
10.1126/science.abb1214

A heterogeneous microbial consortium producing short-chain fatty acids from lignocellulose

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Science **369** (6507), eabb1214.
DOI: 10.1126/science.abb1214

Building niches for multiple microbes

Microbial conversion of heterogeneous feedstocks such as lignocellulose into one or more desired products requires assembly of pathways to both break down the inputs and produce the outputs. Shahab *et al.* assembled a collection of microbes that occupy different spatial niches within a bioreactor and have different metabolic capabilities. In the simplest version of their setup, an aerobic fungus adeptly breaks down cellulose into short sugar chains, an oxygen-tolerant bacterium converts these to lactic acid, and an anaerobic bacterium uses the lactic acid to synthesize the short-chain fatty acid butyric acid. Additional microbes handle limitations in the intermediate stream or divert lactic acid to longer-chain fatty acids, which are potentially more valuable. The integrated production and use of lactic acid for biosynthetic reactions could ideally serve as a platform for biosynthesis using robust, heterogeneous microbial consortia.

Science, this issue p. eabb1214

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