Degradation of the low-calorie sugar substitute 5-ketofructose by different bacteria

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Abstract

There is an increasing public awareness about the danger of dietary sugars with respect to their caloric contribution to the diet and the rise of overweight throughout the world. Therefore, low-calorie sugar substitutes are of high interest to replace sugar in foods and beverages. A promising alternative to natural sugars and artificial sweeteners is the fructose derivative 5-keto-D-fructose (5-KF), which is produced by several Gluconobacter species. A prerequisite before 5-KF can be used as a sweetener is to test whether the compound is degradable by microorganisms and whether it is metabolized by the human microbiota. We identified different environmental bacteria (Tatumella morbirosei, Gluconobacter japonicus LMG 26773, Gluconobacter japonicus LMG 1281, and Clostridium pasteurianum) that were able to grow with 5-KF as a substrate. Furthermore, Gluconobacter oxydans 621H could use 5-KF as a carbon and energy source in the stationary growth phase. The enzymes involved in the utilization of 5-KF were heterologously overproduced in Escherichia coli, purified and characterized. The enzymes were referred to as 5-KF reductases and belong to three different protein families with highly different amino acid sequences, activities, and structural properties. Furthermore, we could show that 15 members of the most common and abundant intestinal bacteria cannot degrade 5-KF, indicating that this sugar derivative is not a suitable growth substrate for prokaryotes in the human intestine.

Key points
- Some environmental bacteria are able to use 5-KF as an energy and carbon source.
- Four 5-KF reductases were identified, belonging to three different protein families.
- Many gut bacteria cannot degrade 5-KF.

Keywords Dietary sugar · Artificial sweetener · Low-calorie sugar · Ketofructose reductase · Human microbiota · Sugar-related disease

Introduction

Several lines of evidence indicate that high sugar intake is a risk factor for the development of obesity, type 2 diabetes, and cardiovascular diseases (Malik et al. 2010; Schulze 2004). In addition, there is an increasing public awareness about the risk of dietary sugars (glucose, sucrose, and fructose) with respect to their caloric contribution to the diet and the rise of overweight of children and adults throughout the world (Schulze 2004; Ludwig et al. 2001). Therefore, low-calorie sugar substitutes are of high interest to replace sugar in foods and beverages. Based on the tendency of increased human sugar intake and the associated negative effects on human metabolism, the demand for alternative sweeteners has increased enormously. Especially naturally occurring low-calorie sweeteners have become the focus of the food industry. Among the already established synthetic sweeteners are saccharin, acesulfame-K, sucralose, aspartame, and neotame (Mattes and Popkin 2009). Disadvantages of these synthetically produced sweeteners can be, for example, expensive synthesis processes, artificial tastes, or undesirable side effects for consumers (Schiffman et al. 1979; Wiet and Beyts 1992). Two

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other non-artificial sugar substitutes used in the food industry are the ketohexose tagatose and the sugar alcohol xylitol. Although both compounds have a high degree of sweetening, they can be partially metabolized by the human metabolism and therefore have a caloric value (Grembecka 2015; Vastenavond et al. 2011). Alternative sweeteners of natural origin are steviol glycosides, which have 30 to 150 times the sweetness of sugar. However, the compounds may have a bitter or licorice-like aftertaste (Soejarto et al. 1982). A promising alternative to natural sugars and artificial sweeteners is the bacterially produced fructose derivative 5-keto-D-fructose (D-threohexo-2,5-diulose, 5-KF). 5-KF is a natural sugar produced by acetic acid bacteria through oxidation of fructose by the membrane-bound enzyme fructose dehydrogenase (Ameyama et al. 1981; Kawai et al. 2013). The compound the bacterially produced fructose derivative 5-keto-D-fructose (5-KF) is a natural sugar providing alternative to natural sugars and artificial sweeteners is rising in popularity. However, the compounds may have a bitter aftertaste and a sweetness comparable to sucrose (Herweg et al. 2018). In addition, 5-KF cannot or can only partially be metabolized by the human organism and thus has a very low-calorie content (Wyrobik et al. 2009). The new potential sweetener has already been detected in various natural foods, including white wine, honey, and elderflower syrup (Burroughs and Sparks 1973; Blasi et al. 2008).

5-KF is produced by the oxidation of fructose by some representatives of acetic acid bacteria of the genus Gluconobacter. Some of the organisms, such as Gluconobacter (G.) japonicus, possess the enzyme fructose dehydrogenase, which can oxidize fructose to 5-KF (Ameyama et al. 1981; Yamada et al. 1996). The genes coding for fructose dehydrogenase were introduced into G. oxydans 621H (Siemen et al. 2018) and the resulting genetically modified strain was capable of producing 5-KF in a fed-batch fermentation process resulting in product titers of up to 490 g L\(^{-1}\) and product yields up to 98% (Herweg et al. 2018). Furthermore, a G. oxydans strain was developed for the efficient production of 5-KF from the cost-efficient and renewable feedstock sucrose (Hoffmann et al. 2020). Before 5-KF can be used as a sweetener, it should be tested whether the compound is degradable by microorganisms and whether it is metabolized by the human microbiota. Therefore, we tested several bacteria for their ability to degrade 5-KF and we analyzed the enzymes involved in this process.

Materials and methods

Materials

All reagents, chemicals, and substrates used in this study were purchased from Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich (Taufkirchen, Germany), or Megazyme (Bray, Ireland). Q5 High-fidelity DNA polymerase, restriction endonucleases, T4 ligase, and PCR reagents were bought from Thermo Fisher Scientific Inc. (Waltham, USA) and New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were synthesized by Eurofins Scientific (Ebersberg, Germany).

Culture conditions and standard molecular techniques

G. oxydans strains, G. japonicus LMG 26773, G. japonicus LMG 1281, and Tatumella (T.) morbirosei DSM 23827 (Table 1) were cultivated at 30 °C and 180 rpm. Precultures of Gluconobacter strains and T. morbirosei were grown in YM-medium (0.6% yeast extract, 0.9% mannitol) and in nutrient broth (0.5% peptone, 0.3% meat extract), respectively. For growth experiments, a medium consisting of 0.6% yeast extract was used. 5-KF was added as indicated. Precultures of Clostridium (C.) pasteurianum were cultivated anaerobically in a complex peptone yeast (PY) medium (Dowell and Hawkins 1974) containing 20 mM glucose in serum flasks under a N\(_2\)/CO\(_2\) (80/20) atmosphere at 37 °C without agitation. Growth experiments with 5-KF were performed using a complex medium with 0.4% yeast extract, supplemented with 48 mM NaHCO\(_3\), 5% modified mineral 3B solution (40 mM KH\(_2\)PO\(_4\), 300 mM NaCl, 4 mM CaCl\(_2\) \(\times\) 2 H\(_2\)O, 2 mM MgCl\(_2\) \(\times\) 6 H\(_2\)O, 1 mM MnCl\(_2\) \(\times\) 4 H\(_2\)O, 0.1 mM CoCl\(_2\) \(\times\) 6 H\(_2\)O, 35 mM Na\(_2\)SO\(_4\)). The pH was adjusted with NaHCO\(_3\). Escherichia (E.) coli DH5\(_\alpha\) was cultivated in lysogeny broth (LB) (Miller 1972) at 37 °C, 180 rpm. Ampicillin (100 μg mL\(^{-1}\)) was added for plasmid maintenance.

HPLC analysis

For the analysis of substrate consumption and product formation analysis, 1-ml samples were taken at different time points and centrifuged at 13,000 \(\times\) g for 1 min. The supernatants were diluted 1:10 with H\(_2\)O. HPLC analysis (Knauer Smartline HPLC system, Knauer GmbH, Berlin, Germany) was performed using an Aminex HPX-87H (300 mm \(\times\) 7.8 mm column (Biorad, Munich, Germany) with 5 mM H\(_2\)SO\(_4\) at 65 °C and a flow rate of 0.6 ml min\(^{-1}\)). Substrates and products were quantified by a refraction index detector (RI detector;Azura RID2.1 L, Knauer GmbH, Berlin, Germany) and a UV detector (Smartline 2600, Knauer, Berlin, Germany) at 210 nm by comparison to calibration curves. For product analysis of 5-KF reduction enzyme assays samples were analyzed by a SpectraSYSTEM HPLC system (Thermo Fisher Scientific Inc., Waltham, USA) using an amino phase column Eurospher II 100 NH2 (250 \(\times\) 3.0 mm; 5 μm particle size) (Knauer GmbH, Berlin, Germany) with integrated precolumn, 90% acetonitrile as solvent, at 40 °C and a flow rate of 0.6 ml min\(^{-1}\). Products were quantified by a refraction index (RI)
Construction of expression systems for \textit{kfr} and \textit{akr}

The gene \textit{kfr} from \textit{T. morbirosei} (HA49_09215) and \textit{sdh} from \textit{C. pasteurianum} (CPAST_c38270), both encoding a putative shikimate dehydrogenase, and the gene \textit{akr} (CPAST_c22030), encoding an aldo/keto-reductase (AKR) from \textit{C. pasteurianum}, were amplified by PCR using Q5 High-fidelity DNA polymerase and the primers KFR_pASK5_for/ KFR_pASK5_rev, SDH_Cpast_for/ SDH_Cpast_rev, and AKR_Cpast_for/ AKR_Cpast_rev, respectively (Table 1). As template genomic DNA of \textit{T. morbirosei} DSM 23827 and genomic DNA of \textit{C. pasteurianum} DSM 525 was used. The genes were cloned into a pASK-IBA.5 vector. The gene \textit{kfr} from \textit{T. morbirosei}
was cloned via the restriction sites EcoRI and XhoI resulting in the plasmid construct pASK5_KFR_TM. The genes sdh and akr from *C. pasteurianum* were cloned via the restriction site BsaI, resulting in the vector constructs pASK5_SDH_Cpast and pASK5_AKR_Cpast, respectively. The plasmids were transformed into competent *E. coli* DH5α cells by heat shock. Positive clones were verified by sequencing.

**Overexpression and purification of the proteins TM-KFR, CP-AKR, GOX0644, and GOX1432**

For protein production, overnight cultures of *E. coli* (5 mL) harboring plasmids of interest were used to inoculate 1 L LB medium and were incubated at 37 °C and 180 rpm in shaker flasks. After reaching an OD600 of 0.4, protein production was induced by the addition of 0.2 μg mL⁻¹ anhydrotetracycline and cells were further cultivated for 4 h. Cultures were harvested at OD600 between 1.0 and 1.5 by centrifugation (9000×g, 4 °C, 15 min). Lysis and purification were performed by sonication as previously described (Kosciow et al. 2016). For protein visualization, polyacrylamide gel electrophoresis was done according to (Laemmli 1970) and protein bands were detected via silver stain as described by (Blum et al. 1987). Analysis of the native conformation of the ketofructose reductases was performed by gel filtration chromatography using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare, Chicago, USA) connected to an AKTA purifier system (GE Healthcare, Chicago, USA). The column was calibrated using the Gel Filtration Calibration Kit HMW (GE Healthcare, Chicago, USA). Equilibration was done with 50 mM Tris-HCl buffer pH 8, containing 150 mM NaCl.

**Measurement of enzyme activities and kinetic parameters**

The reduction of 5-KF and other substrates accompanied by the oxidation of NAD(P)H to NAD(P)⁺ was recorded at 340 nm (ε=6.22 mM⁻¹cm⁻¹). The reaction mixture with a final volume of 1 ml contained 250 μM NAD(P)H, 40 mM potassium phosphate buffer (pH 7), and varying substrate concentrations between 5 and 20 mM. One unit of enzyme activity corresponded to the oxidation of 1.0 μmol of substrate per min and mg protein. The pH optima were determined using the McIlvaine buffer system (McIlvaine 1921) between pH 3.0 and 9.0 containing disodium phosphate and citric acid. Temperature optima were analyzed in a temperature range between 20 °C and 80 °C using standard assay conditions. Nonlinear regressions of Michaelis–Menten data were used to calculate kinetic constants at optimal pH and temperature using 5-KF concentrations between 0.5 and 80 mM and NAD(P)H concentrations between 5 μM and 250 μM, respectively.

Enzyme assays analyzed via HPLC were performed with the same principle as described above. The enzyme assays had a final volume of 250 μL with 5 mM NADPH, 5 mM 5-KF, 40 mM potassium phosphate buffer pH 7 or pH 6 for CP-AKR and 2 μg enzyme. The reaction mixture was incubated at 30 °C or 40 °C for CP-AKR for three hours. The enzyme reaction assays were directly analyzed via HPLC, as described above.

**Transcriptional analysis by qRT-PCR experiments**

Total RNA from *T. morbirosei*, *C. pasteurianum*, or *G. japonicus* LMG 23776 was isolated from 50 mL cultures grown to mid-exponential phase as described by Kröninger et al. (2017). qRT-PCR reactions were performed with a CAPITAL™ qRT-PCR Green Mix One Step Kit (Biotechrabbit, Henningsdorf, Germany), which uses SYBR Green as the fluorescent dye. Each PCR reaction contained 150-200 ng of purified RNA. Temperature cycling and fluorescence measurement was performed with a cycler CFX Connect™ and suitable software (BioRad, Munich, Germany). The ΔΔCt values were calculated by subtraction of the quantification cycle (Ct) of the reference gene ( encoding the 50S ribosomal protein L10 from the Ct value of each gene of interest. The relative change in mRNA abundance was recorded as the ratio of normalized target concentrations (ΔΔCt). The fold change of each gene was calculated using the formula 2^ΔΔCt. The following genes were analyzed: *T. morbirosei* gene HA49_09215 encoding protein KGD75378 (TM-KFR; primers KFR_fw: CGGCGGACC TATGTGAATGG and KFR_rev: GGAAGCAGCACGTA ACAG AG), *T. morbirosei* gene HA49_21310 encoding the 50S ribosomal protein L10 as reference gene (primers T.morbRib50S_fw: TCGTGAAGCTGGCTATACA and T.morbRib50S_rev: TTTGAAACAGGACGACGAG), *C. pasteurianum* gene CPAST_c22030 encoding protein AJA48273.1 (CP-AKR; primers C.past_AKR_fw: CGGAAGGGGTCTGTTCTTATTA and C.past_AKR_rev: TCCCGAGTTCCTTACGCCT T), *C. pasteurianum* gene CLPA_c36850 encoding the 50S ribosomal protein L10 as reference gene (primers: C.pastRib50S_fw: CGTGCAGG CAAATTAGACCTTG and C.pastRib50S_rev: CGCCTTGTA CTAATCCAGGC), *G. japonicus* LMG 26773 gene AD942_05425 encoding protein KXV40663 (GJA0644; primers G.jap-1_KFR-fw: GAACAGATGGACT GCAGAA and G.jap-1_KFR_rev: GATGCATCAACCATGCAT), and *G. japonicus* LMG 26773 gene AD935_10055 encoding the 50S ribosomal protein L10 as reference gene (primers: G.japRib50S_fw: AGAACAGGGTCTGACGGG and G.japRib50S_rev: AGCTTGTCATTGCTTGAG).
Bioinformatic tools

The program Blastp at the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify proteins and to compare amino acid sequences (Johnson et al. 2008). The term similarity is defined as percentage of the number of amino acids that were either identical between the query and the subject sequence or had similar chemical properties. Amino acid sequence alignments were performed with the program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) using default parameters. Kinetic parameters of purified enzymes were determined using nonlinear regression of the Michaelis–Menten data with the program GraphPad Prism Version 9.0.0.

Results

5-KF consumption in aerobic bacteria

It was already shown that T. citrea (former Erwinia citreus) is capable of reducing 5-KF. The organism possesses a 5-KF reductase, which catalyzes the reversible NADPH-dependent reduction of 5-KF to D-fructose (Schrimsher et al. 1988). We tested a close relative, T. morbirosei, for its ability to grow on 5-KF as a carbon and energy source. A complex medium was used for the growth experiments since no mineral medium is available for T. morbirosei and the growth requirements of this organism are unknown. In the control experiment without the addition of a sugar source, the cells grew to an optical density (OD 600) of 1.25 within 20 h (Fig. 1). The doubling time (t_d) was 2.3 h. Cultures with 5-KF revealed a similar growth pattern at the beginning of the experiment (t_d = 1.8 h) with a short slowdown of growth after 16 h. In contrast to the negative control, a second growth was observed and the OD 600 increased to 2.1 (Fig. 1), indicating a typical pattern of diauxic growth. In line with this observation was the fact that the organism consumed significant amounts of 5KF only after 16 h. 5-KF was completely utilized after 25 h and cells turned into the stationary growth phase. It can therefore be assumed that components of the complex medium were first used for growth, as in the control without 5-KF. Only in the second phase, 5-KF was utilized, which was accompanied by an increased OD 600. T. morbirosei was therefore able to use 5-KF as a substrate.

Some species of the genus Gluconobacter are known to produce 5-KF from fructose. The membrane-bound fructose dehydrogenase (Fdh), which is encoded by the fdhSCL genes, plays a crucial role in this process (Kawai et al. 2013). The enzyme oxidizes fructose at the hydroxyl group at position 5, thus forming 5-KF and transferring the released electrons into the respiratory chain of the organism. The resulting 5-KF diffuses via porins in the outer membrane into the extracellular space and accumulates in the culture supernatant. Hence, the question arose whether 5-KF can also be a substrate for growth. For this purpose, the Fdh-producing strain G. japonicus LMG 26773 was tested, which is able to form 5-KF. The organism was grown on a medium containing 0.6% yeast extract and 25 mM 5-KF (Fig. 2). It was found that G. japonicus LMG 26773 consumed large quantities of 5-KF after a lag phase of about 3 h and almost completely converted the substrate after 12 h. The OD 600 increased from 0.05 to 2.2 within 9 h and the doubling time was 1.3 h. The control culture without 5-KF addition showed only an increase in OD 600 from 0.05 to 0.2 (Fig. 2).

The model organism G. oxydans 621H does not possess the key enzyme Fdh for 5-KF production (Siemen et al. 2018).

Fig. 1 Growth and 5-KF consumption in T. morbirosei. Cells were grown in 0.6% yeast extract with 50 mM 5-KF or without 5-KF at 30 °C. (■) 5-KF concentration, (Δ) OD 600 of 5-KF containing cultures, (○) OD 600 of control cultures without 5-KF. The figure shows a representative experiment from three biological replicates (for additional experiments see Fig. S1)

Fig. 2 Growth of G. japonicus LMG 26773. Cells were grown in 0.6% yeast extract with 25 mM 5-KF or without 5-KF at 30 °C. (■) 5-KF concentration, (Δ) OD 600 in the presence of 5-KF, (○) OD 600 in the absence of 5-KF. One out of three independent experiments is shown (for additional information, see Fig. S2)
Therefore, we tested a fdh knock-in mutant of G. oxydans (Hoffmann et al. 2020) for the expression of the fdh genes that allowed the production of the fructose dehydrogenase. Two other strains G. japonicus LMG 1281 and G. japonicus LMG 26773 naturally contained the fdh genes on their chromosomes (gene no. BAM93250 - BAM93252 and KXV40773 - KXV40775, respectively). As expected, all strains grew with fructose as substrate and accumulated 5-KF in the culture supernatant up to a concentration of 50 mM (Hoffmann et al. 2020). In the stationary phase, G. japonicus LMG 1281 and G. japonicus LMG 26773 completely consumed 5-KF within the period 40–98 h and 40–70 h, respectively (Fig. 3). In G. oxydans fdh the degradation of 5-KF was slow and only 10 mM 5-KF were utilized within 60 h.

5-KF consumption in anaerobic bacteria

All 5-KF degrading species described above were strict aerobes and needed O2 as the final acceptor of their respiratory chain. Hence, the question arose whether only aerobic bacteria are able to ferment 5-KF or whether this ability is also observed in anaerobic bacteria. As a model organism we tested C. pasteurianum DSM 525 for its ability to use 5-KF as a substrate. The organism reached an OD600 of 1.7 and a doubling time of 2.0 h with 25 mM glucose as its preferred carbon source. In contrast, C. pasteurianum grew to a final OD600 of 1.4 within 17.5 h in the presence of 23 mM 5-KF, which corresponded to a growth rate of 0.33 h⁻¹ and a doubling time of 2.1 h in the exponential phase (Fig. 4). HPLC analysis revealed a complete consumption of 5-KF after 17.5 h and the synthesis of the expected end products butyrate and acetate, indicating that this compound was metabolized by C. pasteurianum. Without an additional carbon source, C. pasteurianum reached only a final OD600 of 0.15 (Fig. 4).

Since we could show that the anaerobic bacterium C. pasteurianum is able to metabolize 5-KF, the next step was the investigation of whether the main players of the anaerobic human gut microbiota also have the ability to use 5-KF as a carbon and energy source. For consumers and the food industry, these experiments are important as they allow the determination of the effective caloric value of novel and potential sweeteners. Thus, 15 microorganisms were tested as representatives of important members of the human gut microbiota: Agathobacter rectalis DSM 17629, Akkermansia muciniphila DSM 22959, Alistipes shahii DSM 19121, Bacteroides vulgatus DSM 1447, Bifidobacterium adolescentis DSM 20083, Clostridium leptum DSM 753, Collinsella intestinalis DSM 13280, Dorea formicigenerans DSM 3992, E. coli K12, Eubacterium siraeum DSM 15702, Lactobacillus reuteri DSM 17509, Methanomassiliicoccus luminyensis DSM 25720, Parabacteroides johnsonii DSM 18315, Prevotella copri DSM 18205 and Ruminococcus gnavus DSM 108212 (Tab. S1). However, it became evident that the tested organisms did not reach a higher OD600 with 20 mM 5-KF as substrate when compared to the negative control without an additional carbon source (Fig. S5).

Furthermore, HPLC analysis confirmed that 5-KF was not degraded by these bacteria. The results indicated that many important microbial species within the human gut are likely not able to metabolize 5-KF and to produce short-chain fatty acids, which could contribute to the total calorie intake of the human body.

Fig. 3 Degradation of 5-KF in the stationary growth phase by different Gluconobacter strains grown on 0.6% yeast extract and fructose as substrate. (●) G. oxydans fdh, (○) G. japonicus LMG 1281, (□) G. japonicus LMG 26773. The experiment was conducted in triplicate. 5-KF was added to a final concentration of 50 mM. The figure shows a representative experiment from three biological replicates (additional experiments are shown in Fig. S3)
Characterization of 5-KF reductases

As shown above, a couple of bacteria can grow with 5-KF as carbon and energy source. Others were able to use the compound in the stationary phase to ensure survival in their substrate-limited habitat. However, the knowledge about biochemical reactions that are involved in 5-KF degradation is limited. It is known that T. citreua contains a 5-KF reductase that can reduce 5-KF using NADPH as reductant. The corresponding enzyme was purified and characterized (Schrimsher et al. 1988). The N-terminal AS sequence was published and a BLASTx search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) resulted in the identification of protein from T. morbirosei with 94% similarity. Hence, we could identify the corresponding gene (HA49_09215) and amino acid sequence (KGD75378.1). The corresponding DNA fragment was amplified by PCR and the gene was cloned into plasmid pASK-IBA.5, which allowed the precise fusion with the vector encoded Strep-tag II sequence. The protein was produced in E. coli and purified to apparent homogeneity with the help of a streptavidin affinity column (Fig. 5). The protein was referred to as TM-KFR.

As demonstrated by SDS-PAGE (Fig. 5), TM-KFR showed the expected protein band with a molecular mass of 38 kDa. The native protein revealed a single active peak when analyzed by gel filtration. The peak corresponded to 121.3 kDa, indicating that the enzyme is active as a trimer (Table 2). The apparent $K_M$ value of the enzyme for 5-KF, estimated by using several concentrations of the substrate from 0.5 to 40 mM, was $6.4 \pm 0.6$ mM at pH 7.0 and 30 °C (Table 2), while $V_{\text{max}}$ was $1522.8 \pm 141.9$ U mg$^{-1}$. The apparent $K_M$ value for NADPH was $20.4 \pm 11.5$ μM. For a detailed analysis of enzymatic activities, we first determined the substrate spectrum of the protein. It became evident that the enzyme only reduced 5-KF and could only utilize NADPH as cofactor. All other compounds tested (aldehydes, ketones, sugars, keto sugars, sugar acids, polyols, Table 3) were not attacked. Hence, the specificity towards 5-KF can be used to determine the presence and concentration of the alternative sweetener in various foods and beverages in a photometric assay. Such tests were performed and it was found that 5-KF is present in different white wines, honey, and vinegar in micromolar concentrations (not shown).

As shown above, G. oxydans was not able to use 5-KF as carbon and energy source in the exponential growth phase, but consumption of 5-KF was observed in the stationary phase (Fig. 3). To verify which enzymes are responsible for this effect, we tested several oxidoreductases that act on organic compounds containing keto groups for their ability to reduce 5-KF to fructose. All enzymes indicated in Fig. 6 were heterologously produced in E. coli and purified by Strep-tag affinity chromatography. It became evident that the 5-
ketogluconate reducing enzyme GOX1432 (Zahid and Deppenmeier 2016) and the α-ketocarbonyl reductase GOX0644 (Schweiger et al. 2010) were able to reduce 5-KF with NADPH as reductant (Fig. 6). The α-diketone reductase GOX0646 (Schweiger et al. 2013) reduced 5-KF at a very low rate. In contrast, the 2-ketogluconate reductase GOX0417 (Rauch et al. 2010) and the vinyl ketone reductases GOX0502 and GOX2684 (Schweiger et al. 2008) did not reduce 5-KF. The same was true for the α-ketocarbonyl reductases GOX1615 (Schweiger et al. 2010) and GOX0313 (Schweiger et al. 2013) (Fig. 6).

SDS-PAGE analysis showed molecular masses of 34 kDa and 55 kDa for the proteins GOX0644 and GOX1432, respectively (Fig. 5), which were in good correlation with the estimated masses predicted from their amino acid sequences. The native conformation of both enzymes was analyzed via gel filtration, indicating a monomeric structure for protein GOX0644 and a dimeric conformation for protein GOX1432. Kinetic properties of the enzymes with 5-KF as substrate were examined with nonlinear regressions of Michaelis–Menten data. The $K_M$ and $V_{max}$ values of protein GOX0644 for 5-KF as substrate were 4.0 ± 0.8 mM and 32.7 ± 2.1 U mg$^{-1}$ (Table 2). In case of protein GOX1432, a $V_{max}$ value of 230.3 ± 8.0 U mg$^{-1}$ was calculated, which indicates a higher specific activity for 5-KF when compared to GOX0644. In contrast, protein GOX1432 showed a significantly lower affinity for this substrate ($K_M$ value: 42.0 ± 5.4 mM; Table 2). With NADPH as co-substrate of the reaction, $K_M$ values of 61.2 ± 11.4 μM and 19.5 ± 1.0 μM were determined, respectively. The substrate spectra of the enzymes were already published. Protein GOX0644 reduced α-ketoaldehydes, α-diketones, α-keto esters (Table 3), and the

| Table 2 | Properties of 5-KF reducing enzymes |
|---------|-----------------------------------|
| Organism | Enzyme | Monomer [kDa]$^1$ | Conformation | Temp.- optimum $^°C$ | pH- optimum | Specific activity [U/mg] | $K_{cat}$ $^{s^{-1}}$ | $K_M$ 5-KF [mM] | $K_M$ NADPH [μM] |
|---------|---------|-----------------|---------------|-----------------|-------------|----------------------|---------------|--------------|-------------|
| T. morbirosei | TM-KFR | 36.0 | trimer | 30 | 7 | 1522.8 ± 141.9 | 913.9 ± 85.2 | 6.4 ± 0.6 | 20.4 ± 11.5 |
| G. oxydans | GOX0644 | 34.6 | dimer | 35 | 7 | 32.7 ± 2.1 | 18.9 ± 1.2 | 4.0 ± 0.8 | 61.2 ± 11.4$^2$ |
| G. oxydans | GOX1432 | 55.9 | monomer | 30 | 7 | 230.3 ± 8.0 | 214.6 ± 7.5 | 42.0 ± 5.4 | 19.5 ± 1.0$^3$ |
| C. pasteurianum | CP-AKR | 35.9 | tetramer | 40 | 6 | 48.9 ± 7.1 | 29.3 ± 4.3 | 1.3 ± 0.2 | 12.4 ± 3.8 |

$^1$ Molecular mass including Strep-tag
$^2$ Schweiger et al. 2010
$^3$ Zahid and Deppenmeier 2016

Table 3 | Analysis of the substrate spectrum of 5-KF reductases |
|----------------|-----------------------------------|
| Substrates$^1$ | Specific activity [U mg$^{-1}$] |
| Ketones | TM-KFR | GOX0644 | CP-AKR | GOX1432 |
| 2-butanol-3-one | 0 | 0 | 0.2 | 0 |
| 1-phenyl-1,2-propandione | 0 | 71.2 | 5.3 | 0 |
| pentane-2,3-dione | 0 | 20 | 0 | 0 |
| Aldehydes | | | | |
| diacetyl | 0 | 14.3 | 13.5 | 0 |
| D-/L-glyceraldehyde | 0 | 0.1 | 0 | 0 |
| oxalaldehyde | 0 | 1.1 | 0.4 | 0 |
| pyruvaldehyde | 0 | 10.8 | 1.9 | 0 |
| Ketsugars | | | | |
| 5-ketofructose | 1428 | 29.5 | 35.6 | 130 |
| 5-ketogluconate | 0 | 0 | 0.1 | 0.6 |
| L-sorbose | 0 | 0 | 0.2 | 0 |
| Sugars | | | | |
| D-fructose | 0 | 0 | 0 | 17.2 |
| L-sorbosone | 0 | 0 | 0 | 6.8 |

$^1$ Enzyme assays were performed at 30 °C in 1 ml assays containing 40 mM potassium phosphate buffer pH 7 or pH 6 for CP-AKR, 250 μM NADPH and 20 mM substrate as indicated. None of the enzymes could reduce D-arabinose, L-arabinose, glucose, mannose, ribose, tagatose, xylose, maltose, raffinose, sucrose, trehalose, and xylobiose.
sugar derivative 2,5-diketogluconate (Schweiger et al. 2010), which shows structural homologies to the newly discovered substrate 5-KF. Protein GOX1432 was previously described as mannitol dehydrogenase and reduced the sugars D-fructose and L-sorbose as well as the keto sugar 5-keto-D-glucuronate (Table 3) (Zahid and Deppenmeier 2016).

As demonstrated above, *C. pasteurianum* was able to grow on 5-KF. Since the three proteins KFR from *T. morbihonense*, GOX0644 and GOX1432 from *G. oxydans*, were characterized as 5-KF reducing enzymes, Blast analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed to identify corresponding enzymes in *C. pasteurianum*. Two proteins encoding a putative shikimate dehydrogenase (AJA49865.1) and a putative aldo/keto reductase (AJA48273.1) were similar to the KFR from *T. morbihonense* and protein GOX0644, respectively (similarities of 48% and 67%). Both enzymes were produced in *E. coli* and purified via streptavidin affinity chromatography. While the putative shikimate dehydrogenase AJA49865.1 was inactive with 5-KF as substrate, high activity was detected for the aldo/keto reductase AJA48273.1 (referred to as CP-AKR). When analyzed by polyacrylamide gel electrophoresis and silver stain, a single band for CP-AKR could be detected that was in good accordance with the expected size of 35 kDa (Fig. 5). Gel filtration analysis revealed that the native enzyme formed homotetramers. In addition, the kinetic parameters were investigated, indicating a *V*<sub>max</sub> value for 5-KF of 48.9 ± 7.1 U mg⁻¹ and a low *K*<sub>M</sub> value of 1.3 ± 0.2 mM. The *K*<sub>M</sub> for NADPH was 12.4 ± 3.8 μM. The CP-AKR was able to reduce aldehydes as well as different ketones and, additionally, the reduction of 5-ketogluconate and L-sorboson could be shown (Table 3).

Although all described enzymes exhibited 5-KF reductase activity, significant differences were determined concerning the substrate spectrum, the corresponding amino acid sequence, and the tertiary and quaternary structure. To test whether also the reaction mechanism is different, an HPLC analysis of the end products of all four enzymes after 5-KF reduction was performed (Fig. 7). D-fructose and L-sorbose were applied as analytical standards (Fig. 7a). It became evident that protein GOX0644 and its homologous protein CP-AKR produced L-sorbose as main product of 5-KF reduction (Fig. 7c and d). In contrast, protein GOX1432 and TM-KFR from *T. morbihonense*, which did not show sequence similarities, reduced 5-KF to D-fructose (Fig. 7b and e). A closer look at the structure of 5-KF indicates that both prochiral keto groups are homotopic and have therefore the same chemical reactivity. Each sp²-hybridized trigonal planar carbonyl C-atom can be reduced by the enzymes from the re or the si face. Hence, the stereoselectivity of the 5-KF reducing enzymes with respect to the hydride transfer from dehydronicotinamide to the substrate is responsible for the formation of D-fructose or L-sorbose.

**Involvement of the purified enzymes in the metabolism of 5-KF degrading bacteria**

*C. pasteurianum* was able to grow with 5-KF (Fig. 4). Furthermore, the aldo/keto reductase CP-AKR (AJA48273.1) from *C. pasteurianum* showed high 5-KF reducing activity. qRT-PCR experiments revealed that the mRNA abundance of the corresponding gene was increased about 10-fold during growth with 5-KF (Fig. 8), indicating that CP-AKR had an important function in the degradation of this substrate and might be responsible for the formation of fructose, which is then channelled into the central metabolism of *C. pasteurianum*. *G. oxydans* did not use 5-KF as substrate for growth. Therefore, it was not possible to test whether the proteins GOX0644 and GOX1432 were
responsible for 5-KF reduction in vivo. However, in the 5-KF degrading bacterium *G. japonicus* LMG 26773, proteins with high similarity to the 5-KF reducing enzymes GOX0644 and GOX1432 were identified (Fig. S6). These enzymes were referred to as GJA0644 and GJA1432. The corresponding genes were analyzed by qRT-PCR and it was found that the mRNA concentrations were about 4-fold higher in 5-KF grown cells compared to cells, which used fructose as substrate (Fig. 8). The results indicate that the proteins GJA0644 and GJA1432 as counterparts of GOX0644 and GOX1432 are most probably involved in the first step of 5-KF degradation as performed by *G. japonicus* LMG 26773. In contrast to *C. pasteurianum* and *G. japonicus* LMG 26773, a change in the expression pattern of the gene encoding the 5-KF reductase TM-KFR was not observed in *T. morbirosei* (Fig. 8). However, for *T. citrea*, an almost identical 5-KF reductase has already been described and the enzyme was directly connected with the conversion of 5-KF into fructose as substrate for growth (Schrimsher et al. 1988).

### Discussion

In recent years, it has been demonstrated that conventional sugars such as sucrose, glucose, and fructose can promote the development of food-related diseases (Malik et al. 2010; Schulze 2004; Ludwig et al. 2001; Malik et al. 2006; Johnson et al. 2007) such as type 2 diabetes, obesity, and cardiovascular diseases. It can also be observed that consumers are increasingly paying attention to a more conscious diet leading to an increased demand for calorie-free or low-calorie substitutes as an alternative to traditional sweeteners (Marti et al. 2008; Ogden et al. 2006). A promising alternative to natural sugars and artificial sweeteners is the bacteri ally produced fructose derivative 5-KF. Tests with trained taste specialists have shown that the sweetening power and taste are equivalent to the properties of pure fructose. Thus, 5-KF has a stronger sweetening power than sucrose (Herweg et al. 2018; Stone and Oliver 1969). The compound revealed a similar intrinsic sweet threshold concentration in comparison to fructose. Moreover, there is no artificial, bitter or licorice-like aftertaste, which is noticeable when using some other alternative sweeteners such as stevia, saccharin, and acesulfame K (Soejarto et al. 1982; Herweg et al. 2018; Kuhn et al. 2004). In addition, 5-KF is a natural sugar derivative that is found, e.g., in honey, white wine, and vinegar (Siemen et al. 2018), leading to the question of how 5-KF is degraded in nature.
The analysis of hexose interconversion in *Erwinia* species showed that *Erwinia citreus* (now referred to as *T. citrea*) can use 5-KF, and possesses a NADPH-dependent reductase, which catalyzes the reduction of 5-KF to fructose (Schrimsher et al. 1988). Here we show that *T. morbriosei* possesses a very similar enzyme and can grow with 5-KF as carbon and energy source. In nature, 5-KF is produced by *Gluconobacter* species through oxidation of fructose by the membrane-bound enzyme Fdh (Ameyama et al. 1981; Kawai et al. 2013; Yamada et al. 1966; Siemen et al. 2018). The partial oxidation of sugars and polyols is a typical process for *Gluconobacter* species and is referred to as incomplete oxidation (Matsushita et al. 1994). Several sugars and polyols are attacked in this way. The partly oxidized products (e.g., ketogluconates from glucose and L-sorbose from D-sorbitol) are taken up by the cells and are consumed in the second growth phase or allow survival in the stationary phase (Matsushita et al. 1994). To channel the partially oxidized products into the central carbon metabolism, the organisms contain a repertoire of cytoplasmic oxidoreductases that reduce the products, which are then phosphorylated and degraded in the pentose phosphate pathway (Matsushita et al. 1994). The question was whether such a cytoplasmic oxidoreductase also exists for the reduction of 5-KF to fructose, which can be metabolized to gain ATP and intermediates for biosynthesis. Our growth experiments indicated that *Gluconobacter* species, which produce 5-KF naturally, were indeed able to utilize the sugar derivative as a carbon and energy source (Fig. 2).

*Tatumella* and *Gluconobacter* species are microorganisms thriving in aerobic habitats where 5-KF is commonly present due to the fructose dehydrogenase activity of certain *Gluconobacter* spp. Interestingly, we also found that the strictly fermentative anaerobe *C. pasteurianum* can grow with 5-KF as well, though other members of the order Clostridiales (e.g., *Clostridium leptum* and *Agathobacterobacter rectalis*) were not able to utilize this compound (Fig. S5). *C. pasteurianum* mainly uses mono- and polysaccharides as substrates, which are metabolized by glycolysis and converted to butyrate, acetate, and ethanol by butyrate fermentation. Although the organism does not share the same habitats as the strictly oxygen-dependent 5-KF producing *Gluconobacter* spp., it is not unlikely that *C. pasteurianum* is exposed to higher concentrations of 5-KF in nature. As an organism that was isolated from carrot slices and spoiled fruit juices (Dworkin and Gutnick 2012; Feng et al. 2010) *C. pasteurianum* is able to thrive in rotting fruit and vegetable matter, which presumably could have accumulated 5-KF beforehand due to the activity of *Gluconobacter* spp. Hence, it is tempting to speculate that the ability to metabolize 5-KF is not limited to 5-KF producing species but could also be found in bacteria that come in contact with this compound in their natural habitats.

In this context, it is important to understand whether the dietary consumption of 5-KF could also influence the human gut microbiota. As stated above, 5-KF is a potential candidate as an additive in food production for the reduction of high-calorie sugars in processed foods. It is therefore essential to investigate the influence of this compound on the human intestinal flora. The balance in the intestinal flora, which is closely linked to human health, can be shifted by diet. If microorganisms are able to metabolize 5-KF, unlike other representatives of the intestinal microbiota, they will be enriched while the latter group is suppressed. This may affect human well-being and digestion (Autenrieth 2017; Suez et al. 2014). We were able to show that representative microorganisms of the human intestinal tract could not grow on 5-KF as the sole carbon source. Because the selected microorganisms represented the most common and abundant genera in the human intestines, it is unlikely that metabolism of 5-KF occurs within the human gut in considerable amounts. Moreover, since there was also no production of fermentation end-products deriving from 5-KF like, e.g., the short-chain fatty acids propionate, acetate, or butyrate that could contribute to the nutritional value of the sugar derivative (Scheppach 1994), 5-KF can presumably be considered a calorie-free sugar substitute for human dietary applications.

Though we did not find any 5-KF reduction activity in the selected members of gut bacteria, the organisms we found capable of this catalysis were nevertheless phylogenetically rather distant. Unsurprisingly, this also led to the discovery of different enzymes that were responsible for the turnover of 5-KF. Altogether, we were able to identify four enzymes capable of 5-KF reduction that belong to three unrelated enzymatic classes with very different amino acid sequences and structural properties. The first one, TM-KFR from *T. morbriosei*, showed high similarities (94%) to the 5-KF reductase from *T. citrea*, which in turn is related to shikimate dehydrogenases (EC 1.1.1.25) (Schrimsher et al. 1988). In contrast, the second and third enzyme, GOX0644 from *G. oxydans* and CP-AKR from *C. pasteurianum*, belong to the aldo-keto reductase (AKR) superfamily, which show highest similarity to the 2,5-diketo-D-gluconate dehydrogenases from *E. coli* (70%) and *Corynebacterium* sp. (65%) together with the glyoxal reductase from *Bacillus subtilis* (69%). Lastly, protein GOX1432 from *G. oxydans* was previously described as a mannitol dehydrogenase (EC 1.1.1.138). The enzyme is responsible for mannitol-mediated osmoprotection in these organisms and reduces D-fructose to D-mannitol under physiological conditions (Zahid and Deppenmeier 2016). Furthermore, it has the ability to reduce L-sorbose to D-sorbitol and 5-ketogluconate to gluconate (Zahid and Deppenmeier 2016). Thus, protein GOX1432 shows a totally different substrate spectrum than described for KFR from *T. morbriosei* and *T. citrea*. As a basic principle, 5-KF reduction creates either L-sorbose or D-fructose.
depending on the stereoselectivity of the enzymes (Fig. 7). Our hypothesis is that the reduction of the si face of the planar C-carbonyl structure by the enzymes TM-KFR and GOX1432 leads to the formation of D-fructose. In the other case, the keto group is reduced at the re face by the 5-KF reducing enzymes GOX0644 and CP-AKR resulting in the production of L-sorbose.

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Author contribution UD designed the study and wrote the paper. JS conceived, data analysis, and interpretation of the results. All authors analyzed the results and approved the ﬁnal version of the manuscript.

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References

Ameyama M, Shinagawa E, Matsushita K, Adachi O (1981) D-fructose dehydrogenase of Gluconobacter industrius: purification, characterization, and application to enzymatic microbiom determination of D-fructose. J Bacteriol 2:814–823. https://doi.org/10.1128/JB.145.2.814-823.1981

Autenrieth IB (2017) The microbiome in health and disease: a new role of microbes in molecular medicine. J Mol Med 1:1–3. https://doi.org/10.1007/s00109-016-1499-8

Blasi M, Barbe J-C, Dubourdieu D, Deleuze H (2008) New method for reducing the binding power of sweet white wines. J Agric Food Chem 18:8470–8474. https://doi.org/10.1021/jf800665e

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 2:93–99. https://doi.org/10.1002/elps.1150080203

Burroughs LF, Sparks AH (1973) Sulphite-binding power of wines and ciders. 3. Determination of carbonyl compounds in a wine and calculation of its sulphite-binding power. J Sci Food Agric 2:207–217. https://doi.org/10.1002/jsfa.2740240213

Dowell VR, Hawkins TM (1974) Laboratory methods in anaerobic bacteriology, CDC Laboratory Manual. CDC Information Center, Georgia

Dworkin M, Gutnick D (2012) Sergei Winogradsky: a founder of modern microbiology and the ﬁrst microbial ecologist. FEMS Microbiol Rev 2:364–379. https://doi.org/10.1111/j.1574-6976.2011.02999.x

Feng G, Churey JJ, Worobo RW (2010) Thermoaciduric Clostridium pasteurianum spoilage of shelf-stable apple juice. J Food Prot 10: 1886–1890. https://doi.org/10.4315/0362-028x-73.10.1886

Grembecka M (2015) Sugar alcohols—their role in the modern world of sweeteners: a review. Eur Food Res Technol 241:1–14. https://doi.org/10.1007/s00217-015-2458-2

Herweg E, Schopping M, Rohr K, Siemen A, Frank O, Hofmann T, Deppenmeier U, Büchs J (2018) Production of the potential sweetener 5-ketofructose from fructose in fed-batch cultivation with Gluconobacter oxydans. Bioresour Technol 259:164–172. https://doi.org/10.1016/j.biortech.2018.03.038

Hoffmann JJ, Hövels M, Kosciow K, Deppenmeier U (2020) Synthesis of the alternative sweetener 5-ketofructose from sucrose by fructose dehydrogenase and invertease producing Gluconobacter strains. J Biotechnol 307:164–174. https://doi.org/10.1016/j.jbiotec.2019.11.001

Johnson RJ, Segal MS, Sautin Y, Nakagawa T, Feig DI, Kang D-H, Gersch MS, Benner S, Sánchez-Lozada LG (2007) Potential role of sugar (fructose) in the epidemic of hypertension, obesity and the metabolic syndrome, diabetes, kidney disease, and cardiovascular disease. Am J Clin Nutr 4:899–906. https://doi.org/10.1093/ajcn/86.4.899

Johnson M, Zaretskaya I, Raytsev Y, Merezhuk Y, McGinnis S, Madden TL (2008) NCBI BLAST: a better web interface. Nucleic Acids Res 36(Web Server issue):W5–W9. https://doi.org/10.1093/nar/gkn201

Kawai S, Goda-Tsutsumi M, Yakushi T, Kano K, Matsushita K (2013) Heterologous overexpression and characterization of a flavoprotein-cytochrome c complex fructose dehydrogenase of Gluconobacter japonicus NBRC3260. Appl Environ Microbiol 5:1654–1660. https://doi.org/10.1128/AEM.03152-12

Kosciow K, Domín C, Schweiger P, Deppenmeier U (2016) Extracellular targeting of an active endoxylanase by a TolB negative mutant of Gluconobacter oxydans. J Ind Microbiol Biotechnol 7:989–999. https://doi.org/10.1007/s10295-016-1770-6

Kröninger L, Gottschling J, Deppenmeier U (2017) Growth characteristics of Methanomassiliicoccus luminyensis and expression of methyltransferase encoding genes. Archaea 2017:2756573–2756512. https://doi.org/10.1155/2017/2756573. eCollection 2017
