HHS Public Access

Author manuscript

Urology. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Urology. 2015 March ; 85(3): 517–521. doi:10.1016/j.urology.2014.11.013.

Analysis of commercial kidney stone probiotic supplements

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Abstract

OBJECTIVE—To examine the levels of *Oxalobacter formigenes* in probiotic supplements marketed by ™PRO Lab, Ltd, Toronto, Canada, and capsules of Oxalo™ purchased from Sanzyme Ltd, Hyderabad, India, and to measure the ability of these preparations to degrade oxalate *in vitro*.

METHODS—Probiotic supplements and pure cultures of *O. formigenes* were cultured in a number of media containing oxalate. OD_{595} was used to measure bacterial growth and ion chromatography was used to measure loss of oxalate in culture media. *O. formigenes* specific and degenerate *Lactobacillus* primers to the oxalate decarboxylase gene (*oxc*) were used in PCR.

RESULTS—Incubating probiotic supplements in different media did not result in growth of oxalate-degrading organisms. PCR indicated the absence of organisms harboring the *oxc* gene. Culture and 16S rRNA gene sequencing indicated the ™PRO Lab supplement contained viable *Lactococcus lactis* subsp. *lactis* (GenBank accession no. KJ095656.1), while Oxalo™ contained several *Bacillus* species and *Lactobacillus plantarum*.

CONCLUSION—The probiotic supplement sold over the internet by ™PRO Lab, Ltd and Sanzyme Ltd did not contain identifiable *O. formigenes* or viable oxalate-degrading organisms, and they are unlikely to be of benefit to calcium oxalate kidney stone patients.

Keywords

Oxalobacter formigenes; oxalate; probiotic; calcium oxalate kidney stone disease

INTRODUCTION

Calcium oxalate kidney stone patients may consider purchasing kidney stone probiotic supplements sold by ™PRO Lab, Ltd, Toronto, Canada, or Oxalo™, from Sanzyme, Ltd,

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Hyderabad, India. The ™PRO Lab, Ltd website implies the supplement contains the highly efficient, oxalate-degrading, intestinal bacterium, *Oxalobacter formigenes*, while Sanzyme Ltd directly states their Oxalo™ product contains *O. formigenes*. *O. formigenes* is a non-pathogenic, Gram-negative, obligate anaerobic bacterium that commonly inhabits the human gut and degrades oxalate as its major energy and carbon source.[1] Because of the contribution of dietary oxalate to calcium oxalate stone disease, the potential relationship of this organism to intestinal oxalate balance, urinary oxalate excretion and calcium oxalate kidney stone formation has attracted considerable attention.[2] Whether high oral doses of this organism can promote sufficient intestinal oxalate secretion to diminish the oxalate burden on the kidney in individuals with Primary Hyperoxaluria is currently being tested in a clinical trial financed by the biopharmaceutical company OxThera AB (http://www.oxthera.com/). The association between recurrent calcium oxalate stone disease and colonization with *O. formigenes* was assessed in a study of 247 calcium oxalate stone formers and 259 matched controls.[3] The odds ratio for forming a recurrent stone when colonized was 0.3; i.e., a 70% reduction in stone risk. Controlled dietary studies have also indicated colonized individuals excrete lower levels of urinary oxalate[4,5] and have lower levels of plasma oxalate.[5] A review of the colonization frequencies conducted worldwide indicates that 38 – 77% of a normal population is colonized and it was consistently observed that the colonization frequency in calcium oxalate stone formers was about half that in normal subjects.[3,6] Several studies have indicated that the intake of antibiotics can result in the loss of colonization[6–8], and this is supported by lower prevalence of *O. formigenes* in both cystic fibrosis patients[9], and calcium oxalate stone formers who are frequently prescribed antibiotics.[8,10] It is also possible that a lower rate of colonization in stone formers is due to patients restricting dietary oxalate intake. To date, there has only been one study to examine factors that impact colonization, and in this study[6] only a slight (non-significant) trend was observed between prevalence of colonization (simply whether or not a person was colonized with *O. formigenes*) in normal subjects and oxalate intake. The impact of oxalate deprivation on *O. formigenes* colonization warrants further examination.

O. formigenes colonization may prove to be an efficacious and inexpensive method for limiting calcium oxalate stone risk. The goals of this study were to evaluate the levels of *O. formigenes* in kidney stone probiotic supplements sold by ™PRO Lab, Ltd, and Oxalo™, purchased from Sanzyme, Ltd, and to measure the ability of these preparations to degrade oxalate in culture.

MATERIALS AND METHODS

Culture conditions

Pure cultures of *O. formigenes*, strain OxCC13, were grown at 37°C in either Schaedler broth (SBO, BD Biosciences), medium B[1] (an undefined medium with minerals, metals, cysteine, carbonate buffering system, oxalate, acetate, and 0.1% yeast extract), or medium B with 0.5% yeast extract (OXMY). All media were supplemented with 100 mM sodium oxalate and 10 mM sodium acetate.

For determination of colony forming units (CFU) on solid plate medium, a variation of medium B was used that contained 40 mM sodium oxalate, 7 mM $CaCl_2$, 0.5% yeast

extract, 0.1% Na_2CO_3, and 2.0% Bacto agar (OXMC). Plates were incubated in anaerobic containers using GasPak EZ Anaerobe Container System with Indicator (BD Biosciences) sachets at 37°C.

OD_{595} measurements were performed using a Biotek Synergy HT plate reader and pureGrade BRAND*plates*®, 96 well, Transparent, F-bottom.

A single batch preparation of a 60-day supply of the dehydrated kidney stone probiotic supplement (catalog # 1246-87, Lot # 460-04) was purchased November 23, 2013, from ™PRO Lab (http://www.probiotic-lab.com). One 10 × 10 capsules box of Oxalo™ (http://www.nu-division.com/oxalo.html) was purchased April 26, 2014. The expiration date listed on the box of Oxalo™ was June 2015. There was no expiration date listed on the ™PRO Lab supplement. The Oxalo™ product claimed to contain no less than 2.5 billion cells per capsule of *O. formigenes*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Bacillus coagulans*. All culture experiments were completed within three months of purchase date. Culture experiments were conducted in triplicate.

The ™PRO Lab supplement powder (5 mg/ml) was directly added to medium B, SBO, Brain Heart Infusion (BHI, BD Biosciences) and Lactobacilli MRS broth (BD Biosciences) supplemented with 20 mM sodium oxalate. As recommended by the company, 1 g powder was reconstituted overnight in 500 ml sterile water and then a 10% inoculum was added to BHI and MRS without added oxalate, and MRS broth, SBO, and medium B supplemented with 20 mM oxalate. Cultures grown in BHI and MRS were grown without shaking, and cultures grown in SBO, medium B, and MRS containing oxalate were grown in anaerobically sealed serum vials or Hungate tubes. Serial dilutions of overnight sterile water reconstituted cultures were also cultured onto BHI plates containing 2.0% Bacto agar, and L agar plates (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% Bacto agar), and incubated aerobically.

One Oxalo™ capsule (0.3 g) was cultured in 5 ml of anaerobic medium B containing 20 mM sodium oxalate. In some experiments following the addition of the Oxalo™ capsule to the medium, the initial enrichment culture was serially diluted (~10^{-1} to 10^{-6}) in anaerobic 20 mM oxalate medium B. These cultures were incubated at 37°C for 14 days. In addition, a capsule was dissolved in 5 ml of BHI, and 0.5 ml of this culture was added to 10 ml of BHI, SBO, MRS, and these cultures were grown without shaking. A 5% inoculum from the BHI culture was also added to SBO, OXMY, and Schaedler broth containing no oxalate, and these cultures were grown in anaerobically sealed Hungate tubes. For culture on solid medium, the dissolved capsule was plated on a variation of medium B that contained 40 mM sodium oxalate, 0.5% yeast extract, 0.1% Na_2CO_3, and 2.0% Bacto agar. These plates were grown aerobically and in anaerobic containers using GasPak EZ Anaerobe Container System with Indicator sachets.

Oxalate Assay

Following incubation in various media containing 20 mM oxalate, the oxalate content was measured by ion chromatography using an AS22 column, as previously described.[4]

DNA extraction

DNA was extracted from pure cultures of *O. formigenes* using Promega's Wizard Genomic DNA Purification Kit according to the manufacturer's protocol. DNA was extracted from the supplement powder or Oxalo™ capsule using Maxwell DNA Tissue Purification Kit (Promega) according to the manufacturer's instructions.

PCR analysis and sequencing

Primers were designed to amplify a 1284-bp sequence of oxalyl coenzyme A decarboxylase (*oxc*) gene from *O. formigenes* strain OxCC13. The forward primer was oxc7 (5'-ATGTAGAGTTGACTGATGGC-3'), and the reverse primer was oxc4 (5'-AGCCCATACCAATACCCATAAC-3'). Degenerate primers, oxc9 and oxc8 (5'-ATGTATGGTGTTGTMGGYATT-3' and 5'-TCMAMGTAAACACCACCTGGA-3') were also used to amplify *Lactobacillus oxc*.[11] The 16S rRNA gene was amplified with the universal bacterial primers 515F and 805R (5'-GTGCCAGCMGCCGCGGTAA-3' and 5'-GACTACCAGGGTATCTAATC-3'). PCR was performed with GoTaq Green Master Mix (Promega). For sequencing, PCR products were purified using Wizard SV Gel and PCR Clean Up System (Promega) following the manufacturer's protocol. Sequencing of the 16S rRNA gene was done using primer 515F. Sequencing was performed by the Heflin Center Genomics Core Facility (University of Alabama at Birmingham, Birmingham, Alabama).

RESULTS

Culture

Growth curves of pure cultures of OxCC13 are shown in Fig. 1. OxCC13 grew to a higher density at any given time point in SBO compared to OXMY and medium B. OxCC13 growth in medium B reached a lower maximum OD than growth in SBO and OXMY. OxCC13 showed similar specific growth rates during log growth in all media, $\mu = 0.34 \pm 0.01$, equivalent to a doubling time of 2.1 ± 0.1 hours. Plating OxCC13 indicated an OD_{595} of 0.10, close to the end of logarithmic growth, was equivalent to 1.1×10^8 CFU/ml. OxCC13 appeared as translucent, smooth colonies on OXMC plates, and after 3 days the colonies grew to a uniform size of approximately 1 mm in diameter.

To determine whether *O. formigenes* and other oxalate-degrading organisms were present in the probiotic batch preparation obtained from ™PRO Lab, Ltd, the supplement was either directly, or after overnight reconstitution in sterile water, incubated in SBO, BHI, MRS broth, and medium B containing 20 mM oxalate. No growth, as determined by OD_{595}, was observed in any of the media to which powder was directly added, and no growth was observed in SBO and medium B to which overnight reconstituted supplement was added. In contrast, overnight reconstituted supplement incubated in BHI media and MRS broth with and without oxalate showed evidence of bacterial growth. However, IC measurement of media oxalate showed no loss of oxalate in any of the cultures. In contrast, less than 0.1 mM oxalate remained after culture with pure OxCC13 in SBO and medium B.

The overnight reconstituted powder was plated on BHI and L agar plates containing no oxalate. Colonies with similar size and morphology were observed on both plates. The

average CFU/ml from three independent experiments plated onto BHI was 8.8×10^6 (standard error 3.1×10^3). The overnight supplement culture was also plated on OXMC, and after 14 days no colonies or clear zones, which are indicative of oxalate degradation, were visible.

All Oxalo™ medium B cultures (diluted or non-diluted) showed no loss of oxalate after 14 days incubation. Bacterial growth was evident in BHI, MRS, SBO, and OXMY; however, there was no loss of media oxalate.

PCR analysis and sequencing

DNA was isolated from both probiotic supplements and a number of pure *O. formigenes* strains: OxB, BA1, Sox4, OxCC13, OxWR, HOxBLS, OxK, HC1, and OxGP. PCR was performed using both *O. formigenes oxc*-specific primers (Fig. 2A), and degenerate primers designed to detect *oxc* in *Lactobacillus* strains (Fig 2B). All *O. formigenes* strains tested with *oxc*-specific primers exhibited a band of the expected size (1284 bp). Strain OxCC13 was also tested with the degenerate *Lactobacillus* primers and exhibited a band of the expected size (419 bp). However, no PCR product was detected in either probiotic supplement using both *O. formigenes* specific and degenerate *Lactobacillus oxc* primers (Fig 2).

DNA was extracted from ™PRO Lab supplement, and the universal bacterial primers 515F and 805R were used to amplify the 16S rRNA gene. Sequencing of the reaction revealed a PCR product 98% identical to *Lactococcus lactis* subsp. *lactis* (Fig. 3, GenBank accession no. KJ095656.1). In addition, DNA was isolated from an overnight reconstituted supplement culture and from a supplement culture grown in BHI. Sequencing of 16S rRNA genes from these samples also indicated the major viable bacterium in the supplement was *Lactococcus lactis* subsp. *lacti*.

DNA was extracted from an Oxalo™ capsule and from cultures grown in BHI, MRS, SBO, OXMY and Schaedler Broth. Sequencing of 16S rRNA gene indicated *Lactobacillus plantarum* strain Sha 7 was present (GenBank accession no. KF040094.1) Sequencing of 16S rRNA gene from the different cultures identified several *Bacillus* species (Table 1).

COMMENT

Colonization of the intestine with the highly efficient, oxalate-degrading, intestinal bacterium *Oxalobacter formigenes* may reduce the risk of calcium oxalate stone disease. Probiotic supplements that may contain *O. formigenes* are available for purchase over the internet from ™PRO Lab, Ltd and Sanzyme Ltd. Such products would allow both researchers and physicians the opportunity to examine the impact of *O. formigenes* colonization on oxalate balance and recurrence of calcium oxalate stone disease. This study used culture and PCR methods to detect *O. formigenes* within a single batch preparation of each probiotic product. However, results from these approaches indicated the batch preparations contained no *O. formigenes*. Furthermore, cultures in a number of different media containing oxalate, and PCR using degenerate *Lactobacillus* primers that amplify the oxalate decarboxylase gene, both indicated that the preparations lacked bacteria that may

have the capacity to degrade oxalate. Further analysis by 16S rRNA gene sequencing indicated the predominant organism in the ™PRO Lab preparation was *Lactococcus lactis* subsp. *lacti*. This organism has not been reported in the literature to have the *oxc* gene or degrade oxalate in culture. 16S rRNA gene sequencing identified several *Bacillus* species and *Lactobacillus plantarum* in the Oxalo™ capsule. Interestingly, of these species identified in Oxalo™, oxalate decarboxylase is a minor component of the spore coat of *Bacillus subtilis, Bacillus lichenformis* and *Bacillus cereus*.[12] The genome of *Bacillus lichenformis, Bacillus cereus, Bacillus coagulans* contains the oxalate/formate antiporter central to ATP synthesis in *O.formigenes*.[13] Of the 4 stated organisms listed on the Oxalo™ product only *Bacillus coagulans* was detected by 16S rRNA gene sequencing.

This study raises questions about the difficulty of culturing and preparing *O. formigenes* for probiotic use. For example, the procedure recommended by the company to first reconstitute the powder overnight in sterile water may compromise *O. formigenes* viability. A recent randomized, double-blind, placebo-controlled multicenter study showed that ingestion of a lyophilized enteric coated capsulated preparation of *O. formigenes*, Oxabact®, currently not available for purchase, did not result in a significant reduction in urinary oxalate excretion[14], which the authors suggested could have been due to problems with bioavailability of the supplement or viability of *O. formigenes* in this formulation. Thus, studies examining the factors that impact *O. formigenes* survival and revival following processing for probiotic supplement preparation are warranted.

A limitation of this study is only one batch supply of each probiotic supplement was purchased and examined, and it is possible the preparation received was from a bad batch. Nevertheless, the study raises concerns about the efficacy of these products.

CONCLUSION

The probiotic supplements sold by ™PRO Lab, Ltd, and Sanzyme Ltd, claim to contain the oxalate-degrading intestinal bacterium *Oxalobacter formigenes*. However, both culture and PCR methods did not detect the presence of *O. formigenes* in a batch preparation of each product. In addition, the culture and PCR methods used indicated the preparations do not contain other oxalate-degrading organisms. It is our belief that the probiotic supplement sold by ™PRO Lab, Ltd and Oxalo™ purchased from Sanzyme Ltd, will be of little or no benefit to calcium oxalate kidney stone patients.

ACKNOWLEDGEMENTS

This research was supported by NIH grant RO1 DK087967.

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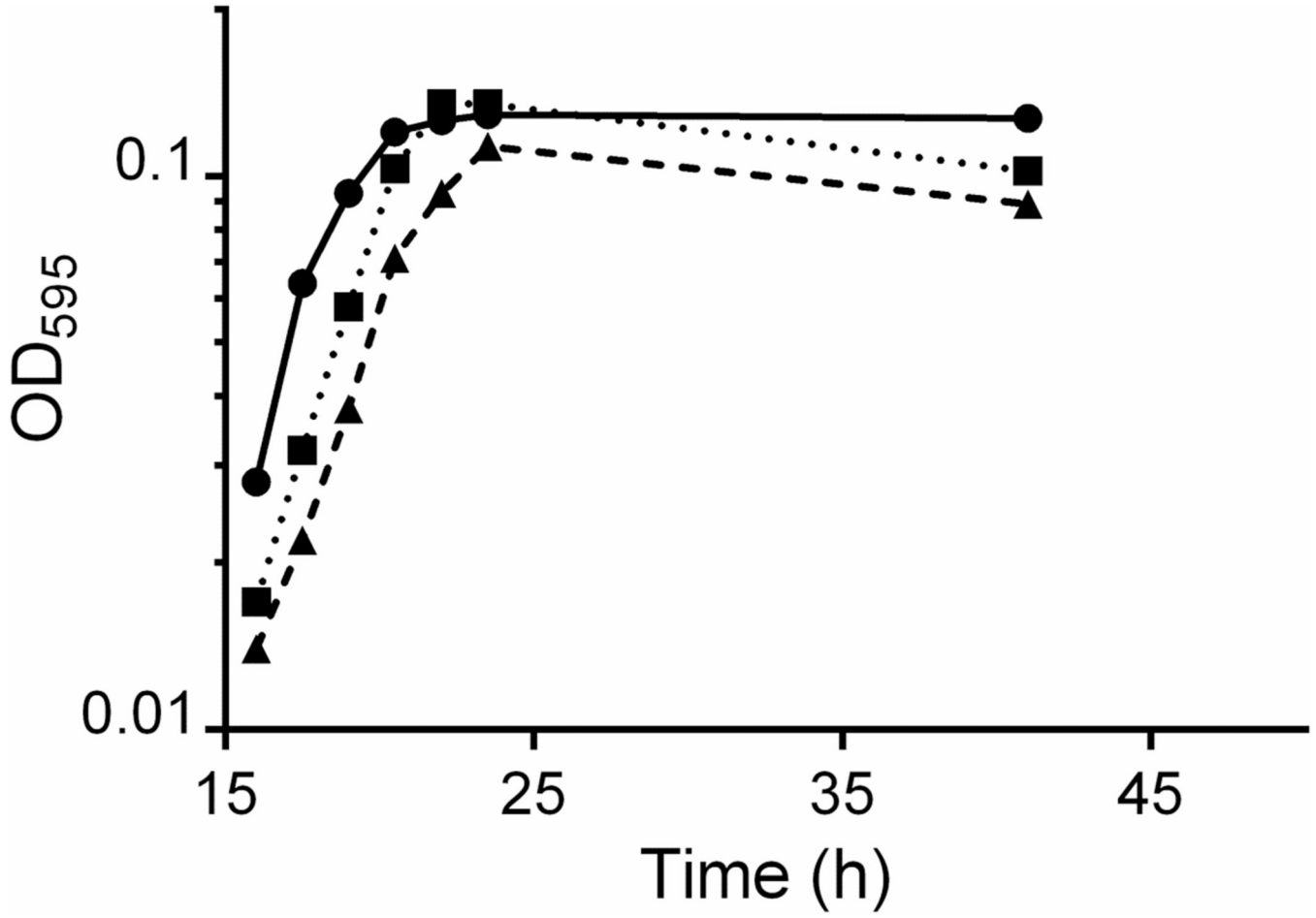

Figure 1.

Growth of *O. formigenes OxCC13*. Representative growth in SBO (●), medium B (- -▲- -), and medium B supplemented with 0.5% yeast (··■··). All media contained 100mM sodium oxalate and 10mM sodium acetate. Starter cultures were grown to end of log growth (OD_{595} 0.1), and then diluted 3/10,000 into each medium to monitor growth over time.

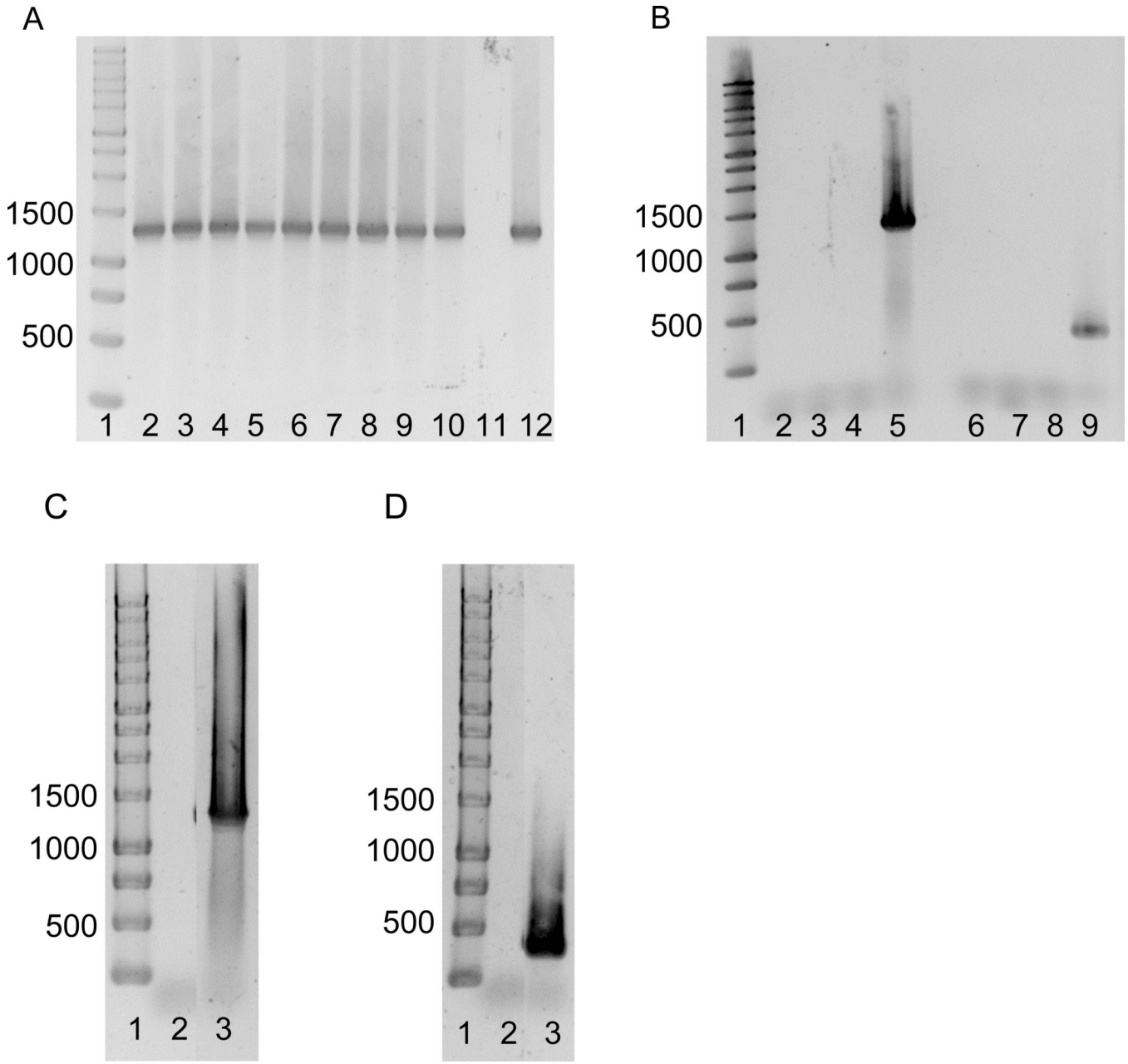

Figure 2.

Amplification of *oxc*.

(**A**) All *Oxalobacter* strains tested with *oxc*-specific primers exhibited a band of the expected size (1284 bp). Lanes: 1, molecular weight ladder (bp); 2, OxB; 3, BA-1; 4, Sox4; 5, OxCC13; 6, OxWR; 7, HOxBLS; 8, OxK; 9, HC1; 10, OxGP; 11, no DNA control; 12, purified DNA from CC13. (**B**) Amplification of *oxc* using *oxc*-specific primers (lanes 2–5) and degenerate *oxc* primers (lanes 6–9, expected size 419 bp) and DNA extracted from ™PRO Lab supplement powder, and BHI cultures of ™PRO Lab supplement. Lanes: 1, molecular weight ladder (bp); 2, BHI ™PRO Lab supplement DNA; 3, ™PRO Lab supplement DNA; 4, no DNA control; 5, purified DNA from CC13; 6, BHI ™PRO Lab

DNA; 7, ™PRO Lab DNA; 8, no DNA control; 9, purified DNA from CC13. (**C**) Amplification of *oxc* using *oxc*-specific primers and DNA extracted from Oxalo™ capsule. Lanes: 1, molecular weight ladder (bp); 2, Oxalo™ capsule DNA; 3, purified DNA from OxCC13. (**D**) Amplification of *oxc* using degenerate *oxc* primers and DNA extracted from Oxalo™ capsule. Lanes: 1, molecular weight ladder (bp); 2, Oxalo™ capsule DNA; 3, purified DNA from OxCC13.

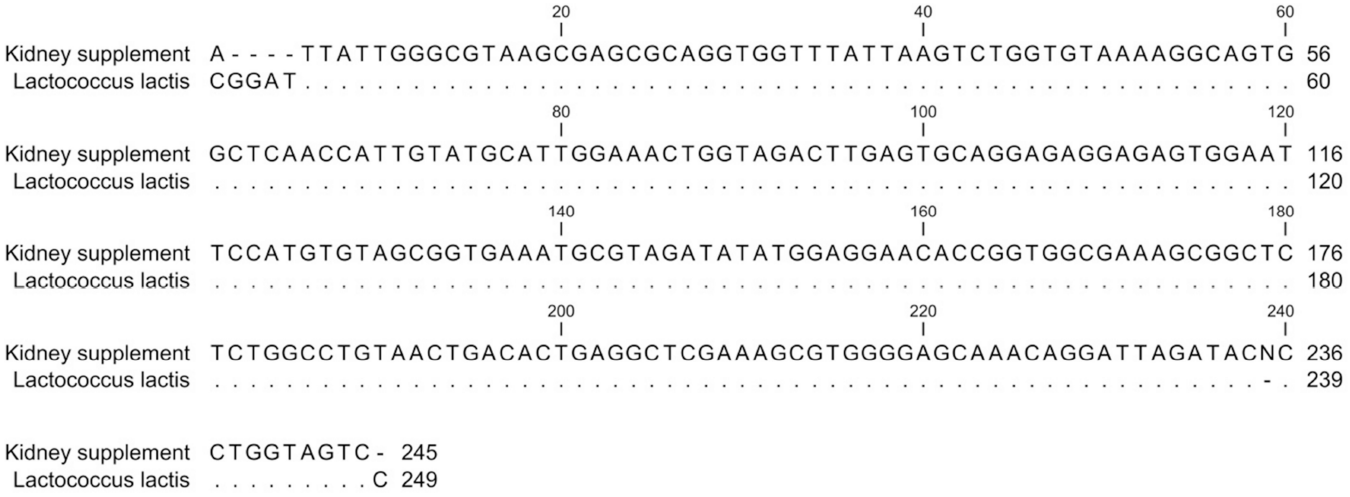

Figure 3.

Sequence alignment of 16S rRNA from ™PRO Lab supplement powder. This PCR product was 98% identical with GenBank accession no. KJ095656.1, *Lactococcus lactis* subsp. *lactis*.

Table 1

Organisms Present in the Probiotic Supplements, ™PRO Lab, “Kidney stone probiotic”, and Sanzyme, Ltd, Oxalo™.

™PRO Lab supplement	GenBank accession no.
Lactococcus lactis subsp. *lacti*	KJ095656.1
Sanzyme Ltd Oxalo™ capsule	
Lactobacillus plantarum	KF040094.1
Bacillus licheniformis	JQ353819.1
Bacillus cereus	KF527826.1
Bacillus coagulans	KJ466151.1
Bacillus subtilis	JQ829568.1