RESEARCH LETTER

Streptomycin use in apple orchards did not increase abundance of mobile resistance genes

Brion Duffy, Eduard Holliger & Fiona Walsh

Federal Department of Economic Affairs, Education and Research EAER, Research Station Agroscope Changins-Wädenswil ACW, Wädenswil, Switzerland

Correspondence: Fiona Walsh, Federal Department of Economic Affairs, Education and Research EAER, Research Station Agroscope Changins-Wädenswil ACW, Wädenswil, Switzerland. Tel.: +41 44 783 6329; fax: +41 44 783 6416; e-mail: fiona.walsh@agroscope.admin.ch

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Abstract

Streptomycin is used as a first-line defense and tetracycline as a second-line defense, in the fight against fire blight disease in apple and pear orchards. We have performed the first study to quantitatively analyze the influence of streptomycin use in agriculture on the abundance of streptomycin and tetracycline resistance genes in apple orchards. Flowers, leaves, and soil were collected from three orchard sites in 2010, 2011, and 2012. Gene abundance distribution was analyzed using two-way ANOVA and principal component analysis to investigate relationships between gene abundance data over time and treatment. The mobile antibiotic resistance genes, *strA*, *strB*, *tetB*, *tetM*, *tetW*, and the insertion sequence IS*1133*, were detected prior to streptomycin treatment in almost all samples, indicating the natural presence of these resistance genes in nature. Statistically significant increases in the resistance gene abundances were occasional, inconsistent, and not reproducible from one year to the next. We conclude that the application of streptomycin in these orchards was not associated with sustained increases in streptomycin or tetracycline resistance gene abundances.

Introduction

In plant agriculture antibiotics are used for the control of plant pathogenic bacteria and have been suggested to increase the likelihood of the selection and spread of antibiotic resistance within the food chain to human pathogens (Vidaver, 2002; Stockwell & Duffy, 2012). Streptomycin was first used in plant agriculture in the 1950s and has been used since then in the prophylactic treatment of fire blight disease in apple and pear orchards in the United States of America (Goodman, 1961). It is also registered for the control of fire blight in Israel, New Zealand, Canada, and Mexico and under restricted conditions in the European Union since 2004 and Switzerland since 2008 due to the perceived environmental and health risks associated with the use of antibiotics in agriculture (Duffy *et al.*, 2005; Stockwell & Duffy, 2012). Fire blight is a destructive bacterial disease of apple and pear trees caused by *Erwinia amylovora,* and streptomycin remains the most reliable and commercially effective control product available against blossom blight stage of fire blight (Norelli *et al.*, 2003). Oxytetracycline is used as the second line of defense against *E. amylovora* infections in the USA, Mexico, and Central America to treat orchards in which streptomycin-resistant *E. amylovora* have been detected (McManus *et al.*, 2002). It has also been frequently used in combination with streptomycin to mitigate pathogen resistance development (Duffy *et al.*, 2005).

Resistance to streptomycin may be mediated by point mutations in the ribosomal protein S12 and the 16S rRNA, encoded by the *rpsL* and *rrs* genes, respectively, or be due to the *strA* (*aph3*), *strB* (*aph6*), and *aadA* (*ant3"*) resistance genes contained on mobile elements (Chiou & Jones, 1993; Finken *et al.*, 1993; McGhee & Jones, 2000; Jones & McGhee, 2001).The insertion sequence element IS*1133* has been frequently located upstream of the *strA-strB* genes on mobile elements, including transposon Tn*5393*, and is thought to increase the expression of these resistance genes (Chiou & Jones, 1993). The *aadA* resistance gene has been most frequently identified in bacteria of animal origin. Tetracycline resistance is conferred by three mechanisms: efflux, ribosomal protection, and in very rare cases, the production of an inactivating enzyme (Roberts, 2005). Tetracycline is currently not authorized

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for use in plant agriculture in the EU. To maintain its efficacy as a second-line antibiotic, we investigated whether the use of streptomycin could either select for bacterial species containing tetracycline resistance genes or aid the selection of the mobile tetracycline resistance genes in the orchard bacteria populations. If either of these possibilities occurred, then increases in the abundances of tetracycline resistance genes would positively correlate with streptomycin treatment. The *tetB* (efflux), *tetM* (ribosomal protection), and *tetW* (ribosomal protection) resistance genes have been identified in the widest diversity of bacterial species and have been located in the environment, animal commensals and pathogens, and human commensals and pathogens (Roberts, 2005; Popowska *et al.*, 2012). The tetracycline resistance genes are frequently associated with plasmids or transposons, which may carry other antibiotic resistance genes (Chopra & Roberts, 2001).

Antibiotic resistance genes contained on mobile elements pose the greatest risk of transfer from agroecosystems or the environment to human pathogens. Controlled experiments comparing antibiotic-treated ecosystems with untreated ecosystems are required to measure the influence of anthropogenic effects on the emergence and spread of these mobile antibiotic resistance genes from the environment to human pathogens. However, approaches that evaluate culturable bacteria for presence of resistance phenotypes and qualitatively determine carriage of specific resistance genes by PCR are limited and in general deal with establishing the qualitative shifts of resistance determinants in response to selection pressures caused by agricultural applications. Studies have demonstrated the presence of both tetracycline- and streptomycin-resistant bacteria and genes within human, animal and environmental bacteria (Karczmarczyk *et al.*, 2011; Mulvey *et al.*, 2013; Walsh, 2013; Walsh & Duffy, 2013). However, as few studies have sequenced the entire mobile elements, it is difficult to assess the impact of the use of streptomycin on the cross-selection of the tetracycline mobile resistance gene. The genomic island Tn*6166* carrying the *strA* and *strB* genes, together with the *tetB* gene mobile elements, has been detected in an *Acinetobacter baumannii* global clone and has been associated with hospital outbreaks of carbapenem-resistant *A. baumannii* (Nigro & Hall, 2012; Saule *et al.*, 2013; Vilacoba *et al.*, 2013). Tetracycline and streptomycin resistance genes have also been detected on plasmids containing the $bla_{\text{CTX-M}}$ extended spectrum β-lactamases in clinical isolates of *Escherichia coli* (Doumith *et al.*, 2012).

The increased concern about the use of antibiotics in plant agriculture has resulted in a number of studies into the effects of antibiotic use on bacterial diversity within these systems (Sundin *et al.*, 1995; Tolba *et al.*, 2002; van Overbeek *et al.*, 2002; Duffy *et al.*, 2011). These studies identified that the bacterial diversity and the proportion of streptomycin or tetracycline resistant bacteria were not altered by the use of antibiotics. The variability of the phyllosphere bacterial community structure over a 3-month period was not linked to streptomycin use (Yashiro & McManus, 2012).

Impact assessment studies of antibiotic application in plant agriculture have focused on bacterial community diversity, qualitative shifts in resistance determinants, or the abundance of resistant bacteria (van Overbeek *et al.*, 2002; Rodríguez *et al.*, 2006; Rodríguez-Sánchez *et al.*, 2007; Binh *et al.*, 2009; Yashiro & McManus, 2012). These studies do not address the complete apple tree ecosystem (flower, leaf and soil) simultaneously, and many do not address the unculturable resistant bacteria within this ecosystem. Laboratory techniques such as culture or PCR are restricted to determining the presence or absence of antibiotic resistance genes or investigate culturable bacteria. Streptomycin and tetracycline are produced naturally by the soil bacteria from different *Streptomyces* species, and thus, bacteria in soil have long since developed resistance to this antibiotic (Pissowotzki *et al.*, 1991; Chopra & Roberts, 2001). Extracting the total DNA and using quantitative PCR overcome the inherent problems of culturing bacteria and enable the analysis of the relative abundances of resistance genes in different agroecosystems.

Using in-house developed multiplex qPCR assays, we aimed to identify whether streptomycin treatment in agriculture impacted on the relative abundances of transferable streptomycin and tetracycline resistance genes in culturable and nonculturable bacteria concurrently from flower, leaf, and soil in apple orchards using nonculture-dependent methods of DNA analysis (Walsh *et al.*, 2011).

Materials and methods

Three experimental orchards were established in apple-producing districts in Switzerland. The apple orchards were not previously treated with antibiotics. Each orchard trial consisted of treatment plots, that is, streptomycin or water sprayed at blossom stage of flowering, separated by nontreated buffer rows of similar size–age apple trees (Fig. 1). All trees were similar-aged Golden Delicious trees to facilitate comparison of results. At Lindau, trees were planted in rows of *c.* 80 m length with 70–75 trees per row. At Güttingen, trees were planted in rows of *c.* 50 m length with 40–45 trees per row. At Wädenswil, trees were planted in rows of *c.* 30 m length with 20–25 trees per row.

Samples were collected at four time points: T1: 1 day prior to streptomycin or water treatment, T2: 2 day after

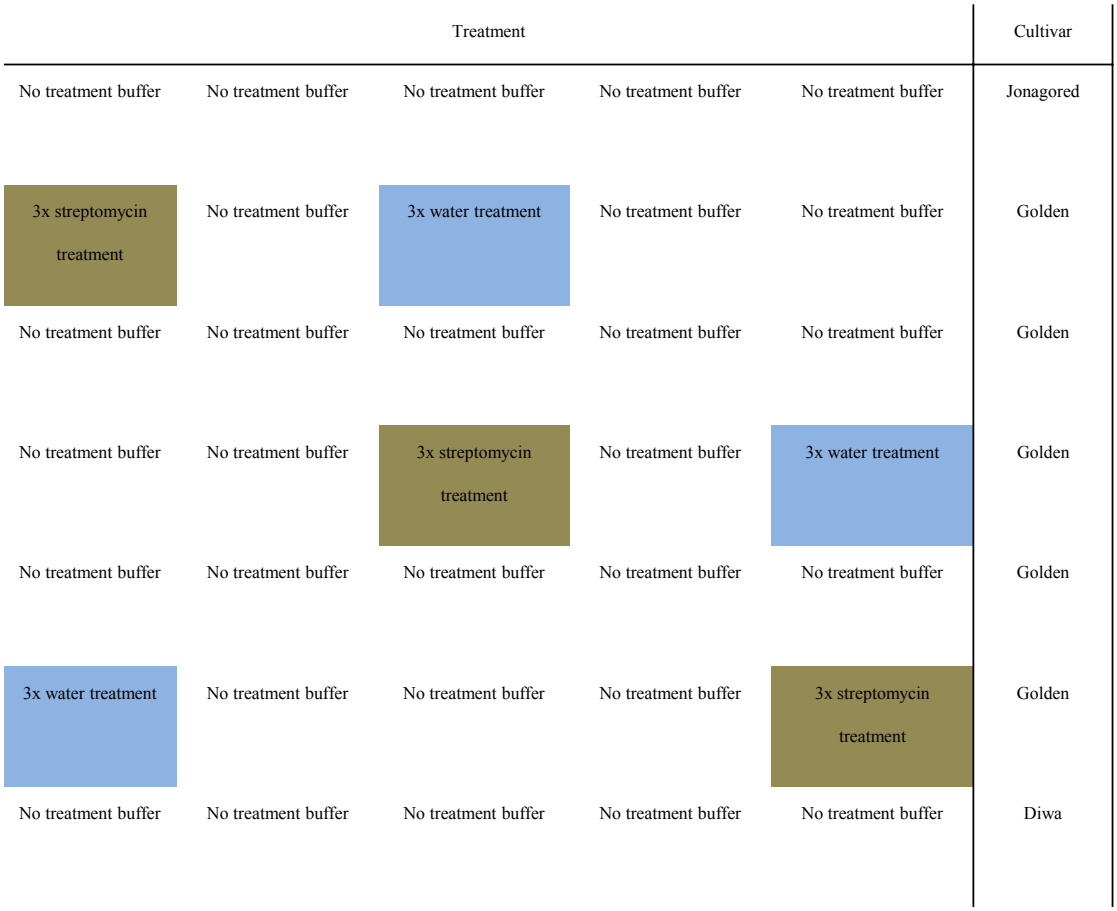

Fig. 1. Schematic diagram of the three orchard sites and streptomycin or water spraying strategies.

streptomycin or water treatment to evaluate immediate impact of treatment, T3: 2 weeks after treatment, and T4: harvest (Fig. 2). Samples were taken at T1 in 2010, 2011, and 2012 to assess the long-term impact of treatment. Samples were taken in 2010 and 2011 at T2, T3, and T4. Samples consisted of reproductive structures (i.e. flowers, developing fruitlets, mature fruit), coronal leaves at the base of flower bushels, and soil under treated trees. Replicate samples were combined from three different apple tree rows for Wädenswil and Lindau apple orchards and in four different apple tree rows for Güttingen apple orchard. Foliar samples consisted of eight randomly collected bushels of blossoms, fruitlets, or fruit, and eight sets of coronal leaves (i.e. the leaves at the base of flower bushels, and thus those leaves with highest exposure to treatments). Blossom stage changed from totally closed at the first sampling date, to completely open at the second sampling date, fruitlets at the third sampling date, and fruit at the final sampling date. At the final sampling date, replicates of eight fruit were picked and placed into clean transparent plastic bags. Soil samples consisted of eight soil cores (10 cm depth) per replicate taken using a stainless steel corer with an internal diameter of 2.5 cm. Soil cores were pooled for each replicate in the field. Pooling of soil cores was used to provide representative samples from replicate orchard plots (Anderson *et al.*, 2003; Gomes *et al.*, 2003; Milling *et al.*, 2004).The samples consisted of three replicates of pooled flower, three replicates of pooled leaf and three replicates of pooled soil samples per time point and treatment (Table 1).

Agricultural formulations of streptomycin sulfate (Strepto, W 6528, Schneiter AGRO AG) were applied at standard agricultural rates (600 g ha^{-1}). Low-drift spraying equipment was used to direct application to the canopy (Duffy *et al.*, 2008). Streptomycin was sprayed during the flower blooming period. Control plots were sprayed with equivalent volume of water.

DNA was extracted from each of the soil samples using the MoBio™ Power Soil® DNA isolation kit (Süd-Laborbedarf GmbH, Gauting, Germany) and as previously described for the flower and leaf samples (Walsh *et al.*, 2011). Leaf, flower, and apple fruit samples were washed

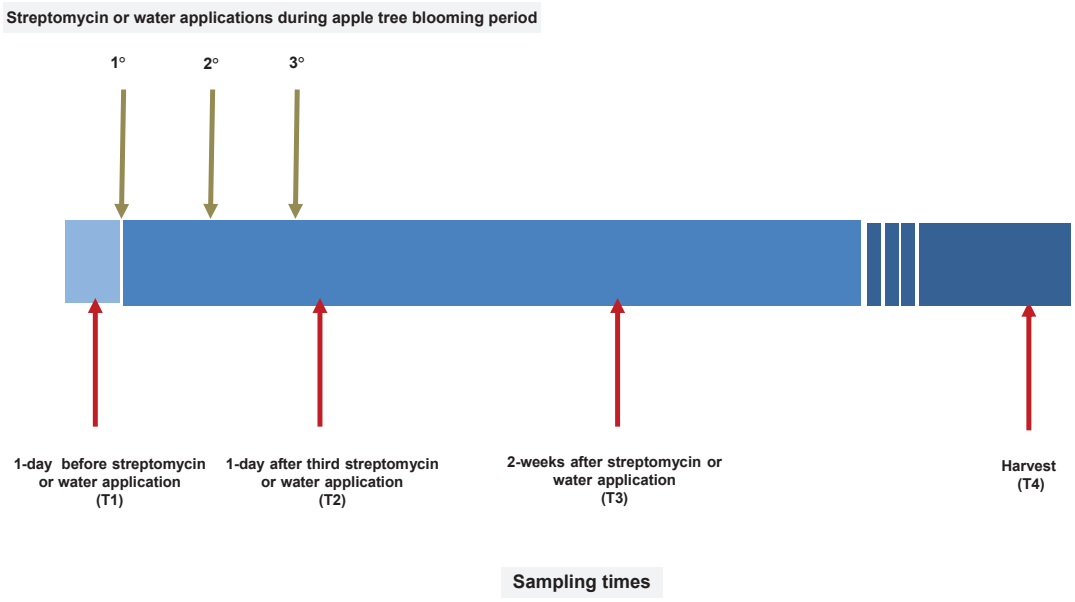

Fig. 2. Diagram of the sampling and spraying schedule of the orchard trees.

in groups of eight samples per sample site in phosphate buffer solution and sonicated for 2 min in a sonicating water bath. DNA was extracted from the washes using the QiagenDNeasy 96 Plant kit (Qiagen AG, Basel, Switzerland). The presence of DNA and absence of PCR inhibitors in all DNA extractions were confirmed by PCR of the 16S rRNA genes(Walsh *et al.*, 2011).

Quantitative monitoring of resistance gene dynamics

The relative quantities of the streptomycin resistance genes *aadA*, *strA*, *strB*, as well as IS*1133* and tetracycline resistance genes *tetB*, *tetM*, and *tetW* in sample DNA were determined using multiplex quantitative real-time PCR (qPCR), as previously described (Walsh *et al.*, 2011). Relative quantification was performed using the delta–delta Ct method (Livak & Schmittgen, 2001). The relative quantity (RQ) of the genes was calculated using the comparative Ct program in the Applied Biosystems software. The RQ of antibiotic resistance genes present in the positive control samples was automatically set to one by the Applied Biosystems 7500 software. All of the samples were then compared with the quantity of antibiotic resistance gene in the positive control. When the antibiotic resistance gene was not detected in the sample no RQ value was calculated. The function of the endogenous control was to normalize the quantity of DNA in each of the samples (Hardwick *et al.*, 2008).The relative quantities of the resistance genes were normalized using the 16S rRNA gene abundances, thus ensuring that the RQ values of the target antibiotic resistance genes were not biased by differences in the quantity of bacteria or DNA present in the samples.

Statistical analyses

Statistical analysis was performed using XLSTAT 2011 software. Data distribution was analyzed using two-way analysis of variance (ANOVA) statistics to investigate relationships between gene abundance data based on year, treatment, and sampling time. All analyses were set at a significance level of $P < 0.05$, indicating a statistically significant difference in the gene abundance in the samples. The variability within the data sets and correlations between different factors were analyzed using principal component analysis (PCA) statistics within XLSTAT.

Results and discussion

This study is the first to compare the RQ of streptomycin resistance and tetracycline resistance genes in comparable streptomycin treated and control apple orchards. We performed sufficient repetitions to enable statistical analyses and comparison. Previous studies, which have measured the effects of antibiotic use in agriculture, have predominantly focused on culturable bacteria or the presence of antibiotic resistance genes or plasmids or on the effects on the bacterial diversity (Sundin *et al.*, 1995; Tolba *et al.*, 2002; van Overbeek *et al.*, 2002; Rodríguez *et al.*, 2006; Rodríguez-Sánchez *et al.*, 2007; Binh *et al.*, 2009; Duffy *et al.*, 2011). Culture-based studies are limited to

Table 1. Samples obtained per time point for each orchard and treatment

Orchard Time point 1	Orchard Time point 2	Orchard Time point 3	Orchard Time point 4
Flower Water R1	Flower Water R1	Flower Water R1	Apple Water R1
Leaf Water R1	Leaf Water R1	Leaf Water R1	Leaf Water R1
Soil Water R1	Soil Water R1	Soil Water R1	Soil Water R1
Flower Water R2	Flower Water R2	Flower Water R2	Apple Water R2
Leaf Water R2	Leaf Water R2	Leaf Water R2	Leaf Water R2
Soil Water R2	Soil Water R2	Soil Water R2	Soil Water R2
Flower Water R3	Flower Water R3	Flower Water R3	Apple Water R3
Leaf Water R3	Leaf Water R3	Leaf Water R3	Leaf Water R3
Soil Water R3	Soil Water R3	Soil Water R3	Soil Water R3
Flower Strep R1	Flower Strep R1	Flower Strep R1	Apple Strep R1
Leaf Strep R1	Leaf Strep R1	Leaf Strep R1	Leaf Strep R1
Soil Strep R1	Soil Strep R1	Soil Strep R1	Soil Strep R1
Flower Strep R2	Flower Strep R2	Flower Strep R2	Apple Strep R2
Leaf Strep R2	Leaf Strep R2	Leaf Strep R2	Leaf Strep R2
Soil Strep R2	Soil Strep R2	Soil Strep R2	Soil Strep R2
Flower Strep R3	Flower Strep R3	Flower Strep R3	Apple Strep R3
Leaf Strep R3	Leaf Strep R3	Leaf Strep R3	Leaf Strep R3
Soil Strep R3	Soil Strep R3	Soil Strep R3	Soil Strep R3

Time point 1 was 1 day prior to streptomycin or water treatment. Time point 2 was 1 day after streptomycin or water treatment. Time point 3 was 2 weeks after streptomycin or water treatment. Time point 4 was harvest.

Strep, sprayed with streptomycin; Water, sprayed with water; R, row replicate of apple trees within the orchard.

only a small proportion of the microbiome in a given environment (Staley & Konopka, 1985; Amann *et al.*, 2001). The mere detection of antibiotic resistance genes in the case of streptomycin use in agriculture does not analyze a cause-and-effect link between streptomycin use and antibiotic resistance gene presence, as streptomycin is produced naturally by soil bacteria and thus is a natural component of the soil bacteria.

To quantify the baseline RQ value for each resistance gene in each sample, the RQ values of each orchard were measured at T1, prior to treatment in all orchard samples (Supporting Information, Table S1). The resistance genes, with the exception of *aadA*, were detected in almost all of the flower, leaf, and soil samples, from each orchard at T1. The *aadA* gene was detected in 15 different samples. This indicates that all of the streptomycin and tetracycline resistance genes were present prior to streptomycin application and highlights the importance of analyzing the relative abundances of the resistance genes in comparison with detecting their presence or absence.

The relative quantities of the resistance genes in the streptomycin-treated samples were compared with their equivalent control samples (treated with water) using ANOVA statistical analysis. There were no statistically significant changes in the resistance gene abundances between the streptomycin-treated and control samples from the first sample taken in year 2010 and those taken at T1 in 2012, identifying that there were no long-term effects of the use of streptomycin on resistance gene abundances, where statistical significance corresponded to a P value < 0.05. In the flower samples, there was a statistically significant increase in RQ values for *strA* and *strB* in all three orchards treated with streptomycin at 1 day after treatment (T2) or 2 weeks after treatment (T3) in 2010 (Table 2). At harvest (T4), these RQ values returned to levels similar to prestreptomycin treatment levels and did not increase significantly in 2011.

There were no significant increases in *aadA* resistance gene abundance in any sample in any year associated with streptomycin treatment. In 2011, there was a significant increase in IS*1133* at harvest (T4) within the streptomycin treated flower samples from Lindau. In the streptomycin-treated leaf samples from Güttingen in 2010, there was a significant increase in abundance of *strA* at 1 day after treatment (T2).This was the only significant increase in *strA* abundance in streptomycin treated leaf samples. In

Table 2. Statistically significant increases in mobile streptomycin resistance gene abundance in streptomycin-treated samples in comparison with equivalent water-treated samples

Orchard	Genes	Year	Time point	Sample type	*P* value
Wädenswil	*strA*	2010	T3	Flower	0.017
Wädenswil	*strB*	2010	T3	Flower	0.004
Wädenswil	*strB*	2011	T3	Leaf	0.034
Lindau	*strA*	2010	T3	Flower	0.041
Lindau	*strB*	2010	T3	Flower	0.026
Lindau	IS*1133*	2011	T4	Flower	0.011
Güttingen	*strA*	2010	T2	Flower	0.029
Güttingen	*strB*	2010	T2	Flower	0.019
Güttingen	*strA*	2010	T2	Leaf	0.037
Güttingen	IS*1133*	2011	T4	Leaf	0.025
Güttingen	IS*1133*	2011	T4	Soil	0.021

2011, there was a significant increase in abundance of *strB* at 2 weeks after streptomycin treatment (T3) in the Wädenswil leaf samples from the streptomycin treated orchard samples. There were increases in the abundances of IS*1133* gene at harvest (T4) in the Güttingen streptomycin-treated leaf and soil samples in 2011. There were no statistically significant increases in *strA* or *strB* gene abundance at harvest (T4), in any streptomycin-treated sample. There were also no increases in streptomycin resistance gene abundance in any soil sample.

The statistically significant increase in tetracycline resistance gene abundance in the streptomycin-treated samples in comparison with their equivalent controls was detected predominantly in 2011. There was only one statistically significant increase in the RQ values of the streptomycin treated samples: *tetM* abundance in the streptomycin-treated Güttingen soil samples from 2010 2 weeks after streptomycin treatment (T3) (Table 3). The resistance gene abundance reduced to levels similar to the control samples at harvest (T4) and remained at background levels at the sampling time point of 1 day prior to treatment (T1) of the following year (T1 of 2011). In 2011, there was a significant increase in *tetM* abundance at 1 day after treatment (T2) within the streptomycin-treated flower samples from Güttingen and in *tetB* and *tetW* at harvest (T4) within the streptomycin-treated flower samples from Lindau. In 2011, there was a significant increase in abundance of *tetB*, *tetM,* and *tetW* at harvest (T4) in the streptomycin-treated leaf samples from the Wädenswil orchard. There were increases in the abundances of *tetB* gene at harvest (T4) in the leaf and soil samples from the streptomycin-treated sections of the Güttingen orchard in 2011. There were also increases in the abundances of *tetM* in soil samples from the streptomycin-treated samples of the Güttingen orchard in 2010.

These increases in antibiotic resistance gene abundances were sporadic and thus may have been influenced by changes in the microbial populations on the flowers and leaves as the morphology of the flower changed from flower to applet to apple and from young leaf to old leaf. The flower and leaf microbiomes are poorly understood, and studies of the changes that occur within the flower or leaf microbiomes are rare (Shade *et al.*, 2013). A study of the phylogenetic diversity of the microbiome of flowers sprayed with streptomycin in comparison with unsprayed indicated that there was slightly lower microbial diversity on the streptomycin-treated flowers in comparison with the controls (Shade *et al.*, 2013). However, this study analyzed the microbial diversity within the first week of the flower, and our study was performed from flower opening to apple, a timeframe of *c.* 4–5 months. The sporadic increases in resistance genes on the flowers and the differences between the different sites could be due to differences in the growing rates of the flowers and leaves over this timeframe. Other influences such as the level of insects and distance from other farms and sources of antibiotic-resistant bacteria could also play a role, but is extremely difficult to quantify. Changes in soil temperature or soil moisture may also have played a role in the changes in bacterial diversity and thus antibiotic resistance gene abundance.

Table 3. Statistically significant increases in mobile tetracycline resistance gene abundance in streptomycin-treated samples in comparison with equivalent water-treated samples

Orchard	Genes	Year	Time point	Sample type	*P* value
Wädenswil	*tetB*	2011	T4	Leaf	0.001
Wädenswil	*tetM*	2011	T4	Leaf	0.014
Wädenswil	*tetW*	2011	T4	Leaf	0.001
Lindau	*tetB*	2011	T4	Flower	0.034
Lindau	*tetW*	2011	T4	Flower	< 0.0001
Güttingen	*tetM*	2010	T3	Soil	0.048
Güttingen	*tetM*	2011	T2	Flower	0.004
Güttingen	*tetB*	2011	T4	Leaf	< 0.0001
Güttingen	*tetB*	2011	T4	Soil	< 0.0001

There was no significant, repeated impact of streptomycin application on streptomycin resistance genes in orchard regardless of year, location, time, or samples type. A direct influence of the streptomycin treatment would be expected to result in changes in the abundances of the *strA* and *strB* genes, conferring streptomycin resistance. However, while these genes were present in all samples, the use of streptomycin did not maintain a higher abundance of these resistance genes in the treated orchard samples in comparison with the controls. The fluctuations in the statistically significant increases in the abundances of these resistance genes were sporadic and were not repeated, either within orchards over time, between orchards or sample types. The abundances of these resistance genes at the first time point (T1) at 2011 and 2012 were not statistically significantly higher than in 2010, indicating that there was no long-term impact of streptomycin use on streptomycin resistance gene abundances.

In addition to the resistance genes, *strA* and *strB*, frequently associated with transferable streptomycin resistance, we also sought to identify whether streptomycin use was associated with changes in the rare resistance gene *aadA* and the insertion sequence (IS*1133*) associated with *strA* and *strB* genes on mobile elements. The *aadA* gene was rarely identified in all samples, and there was no association with streptomycin treatment. The IS*1133* gene was frequently detected in high relative abundance but was not associated with streptomycin use. We suggest that this may be due to the presence of a diverse number

of insertion sequences in environmental bacterial DNA, with similar genetic sequences to IS*1133*.

The tetracycline resistance genes were present in all sample types, years, and both streptomycin-treated and control samples. There was no significant association of increased tetracycline resistance gene abundance with streptomycin treatment. Tetracycline and streptomycin resistance genes have rarely been identified on the same mobile element, which may explain the lack of increased abundance in tetracycline resistance genes (Srinivasan *et al.*, 2008; Wibberg *et al.*, 2013). However, bacteria resistant to both streptomycin and tetracycline have been frequently identified in the environment, food, and human pathogens dating back to 1962 (Datta, 1962; Popowska *et al.*, 2012; Tadesse *et al.*, 2012).

There were variations between the RQ values of 2010 for all samples and those of the following years (Table S1). The RQ values of all genes were in general greater in 2010 than the following years and ranged from 0 to 3.4×10^6. The RQ values of all genes in the samples from 2011 and 2012 were many folds lower in general and ranged from 0 to 44.5. The variations were consistent between water treated and streptomycin treated and were not significantly influenced by the use of streptomycin, the sample type, or orchard location. The only difference that we can identify between 2010 and 2011 or 2012 is the weather in May 2010. The weather is an important factor in the colonization of apple tree blossoms with the pathogen *Erwinia amylovora* and thus may also be an important factor in the variation of the antibiotic-resistant bacterial populations. The weather in May 2010 was between 1.5 °C and 2 °C colder than normal and was wet and cloudy (MeteoSchweiz, www.meteoswiss.admin.ch). However, the weather in May 2011 and May 2012 was between 1.5 °C and 8 °C warmer than normal (MeteoSchweiz). We cannot definitely link the temperature to the variations in RQ values, but warm, wet weather is the optimal condition for infection of apple blossoms with *E. amylovora* and thus play a role in the bacterial ecology of the apple tree (Johnson, 2000). Thus, it is possible that the lower than normal temperature in May 2010 was advantageous to the antibiotic-resistant bacteria in comparison with those in 2011 and 2012.

PCA was performed to compare the variability of the RQ values of the entire data set and within the selected components, that is, sample type, year, and each of the resistance genes (Fig. 3 and Table S1). Individual PCA graphs are displayed in Figures S1 to S8. Each data point is one of a set of three replicates. In the analysis of the total data set, almost all data points cluster together,

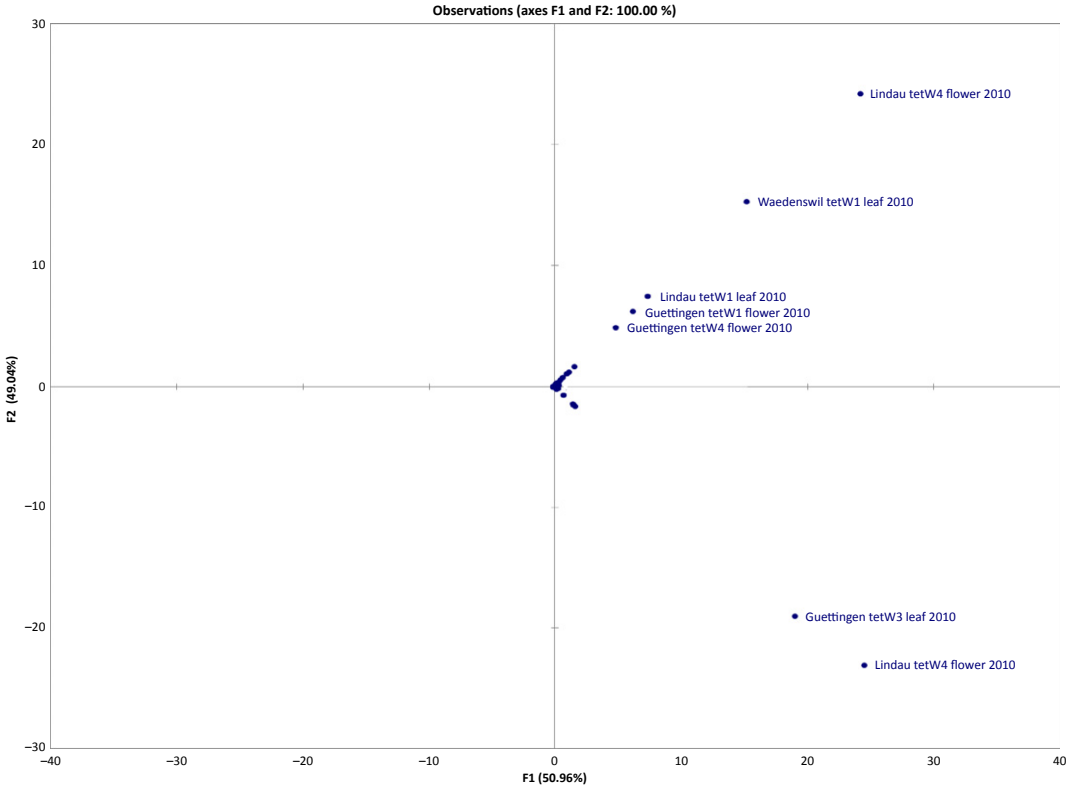

Fig. 3. PCA of the entire data set. The RQ values are clustered according to similarity.

indicating little variances within the results as a whole. The outliers of this data set comprised single RQ values for *tetW* from flower or leaf samples from 2010, which do not form additional clusters. The PCA of the streptomycin resistance genes *strA* and *strB* indicates homogeny between the RQ values. There were four RQ values of a total of 524, which did not cluster in the main group from Lindau and Güttingen orchards from 2010. There was greater variability in the tetracycline resistance gene RQ values, but again the majority of RQ values clustered together. The outliers were from each orchard and from leaf or flower samples. The PCA indicated that the variability within the RQ values was due to specific values and not clusters of values. Therefore, the few outlying values were not associated with specific orchards, sample types or resistance genes.

The results of this study indicate that the controlled use of streptomycin in apple orchards for the control of fire blight disease did not increase the abundance of mobile streptomycin or tetracycline resistance genes. Previous studies have sought to investigate the effects of antibiotic use in agriculture, including the use of streptomycin in apple orchards on bacterial diversity. These studies have identified no effect on bacterial community diversity or the percentage of resistant bacteria (Tolba *et al.*, 2002; Rodríguez *et al.*, 2006; Rodríguez-Sánchez *et al.*, 2007; Binh *et al.*, 2009; Yashiro & McManus, 2012). However, as we have demonstrated, the streptomycin and tetracycline resistance genes are present in almost all of the apple orchard soil, phyllosphere, and flower microbiomes prior to streptomycin treatment. Therefore, the presence or absence of these resistance genes is not sufficient to identify a cause-and-effect link between streptomycin use in apple orchards and increasing antibiotic resistance. Studies that investigated the proportion of resistant culturable bacteria were not complete, as the unculturable proportion of bacteria were not investigated. Although these studies have shed some light on the impact of streptomycin use in agriculture, they are limited. Our study overcomes these limitations by measuring the relative quantities of resistance genes in the total bacterial population. We have also concurrently examined samples from flowers, leaves, and soils in replicated streptomycin-treated and control orchards, enabling statistical analyses of the results.

The restrictions applied to the use of antibiotics in agriculture are predominantly based on studies performed in animals, rather than plants. The concerns surrounding antibiotic use are understandable as antibiotic use in animal production and human medicine have selected for increased levels of resistance. However, our study identified the lack of selection of the investigated transferable streptomycin or tetracycline resistance genes in apple orchards associated with streptomycin application. Streptomycin is currently the only treatment for fire blight in apple growing countries within Europe. Until alternative solutions are found, it is an essential component of the management strategy of apple orchards where the disease is present. Our study expands on a growing body of evidence, which concludes that restricted streptomycin use in apple orchards for the control of fire blight has a low impact on antibiotic resistance selection.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Relative quantity (RQ) values, averages and standard deviations of antibiotic resistance gene abundances in all samples in each year.
Fig. S1. RQ values of *aadA*.
Fig. S2. RQ values analyzed according to sample types.
Fig. S3. RQ values of *strA*.
Fig. S4. RQ values of *strB*.
Fig. S5. RQ values of *tetB*.
Fig. S6. RQ values of *tetM*.
Fig. S7. RQ values of *tetW*.
Fig. S8. RQ values analyzed according to year.