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Full Length Research Paper

Effect of *Piper nigrum*, *Thymus vulgaris* and *Syzigiuim aromaticum* essential oils on the microbiological and sensory quality of Pork sausages during preservation

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The use of essentials oils (EOs) as alternative of nitrites and nitrates in sausages will be proposed in this study. For this purpose, the minimal inhibitory concentration (MIC) of white *Piper nigrum* of *Penja*, *Thymus vulgaris* and *Syzigiuim aromaticum* EOs were determined *in vitro* against *Escherichia coli* ATCC25922, *Salmonella enteritidis* 155A and *Staphylococcus aureus* NCTC10652. It follows from this study that, *T. vulgaris* EO was active on *E. coli* ATCC25922, *S. aureus* NCTC10652 and *S. enteritidis 155A* with MICs of 312.5, 625 and 1250 ppm, respectively. As for *S. aromaticum* EO, it reduced the same germs with MICs of 1250, 2500 and 5000 ppm, respectively. The combination of MICs of 1250 (*T. vulgaris*) and 5000 ppm (*S. aromaticum*) was selected (CMICR) for its bactericidal effect on 03 pathogens. The CMICR totally reduced *E. coli* ATCC25922 and *S. enteritidis* 155A after 3 days of storage. The sensory analysis shows that SAUS 324 and SAUS 589 produced with white *P. nigrum* of *Penja* were appreciated by the panelists. The combination of CMICR and white *P. nigrum* of *Penja* can be considered as an alternative to chemical preservatives to limit the growth of bacteria and improve the sensory characteristics of sausages.

Key words: Sausages, *Piper nigrum*, *Penja*, *Thymus vulgaris, Syzigiuim aromaticum*, antibacterial activity.

INTRODUCTION

The transformation of meat is a process that has existed since ancient times. It is based on the use of a set of processes that lead to the modification of the texture and appearance of the meat with the main objective of increasing the shelf life of the products thus processed (Toldrá et al., 2021). The unitary operations applied to this raw material often include grinding, smoking, salting, fermenting and cooking which lead to a wide range of products including blood sausage, andouilles, ham, pate, potted meat and sausages (Durand, 1999). Among the products mentioned earlier, fresh sausages are the most consumed and appreciated meat products in the world (Šojić et al., 2019). However, sausages are highly susceptible to microbial contamination because of their

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high protein content (Azad et al., 2022; Fursik et al., 2018). They are favorable to the development of altering and pathogenic microorganisms which are responsible on one hand for the marketable quality (taste, smell, appearance) and on the other hand for food-borne diseases such as food poisoning and intoxication (Bailly et al., 2012).

In order to solve this problem of microbial contamination, meat companies have developed several preservation methods such as the use of sulfites, nitrites and nitrates (Nair et al., 2020). However, although they improve the sanitary and organoleptic quality of meat products, these chemicals can cause health problems. High levels of nitrates and nitrites in meat products caused an estimated 600,000 deaths in Germany in the early 20th century (Honikel, 2008). Several studies have also shown that nitrites have a harmful effect on human reproduction (Manassaram et al., 2006). In addition, the International Agency for Research on Cancer (IARC) has recently concluded that the ingestion of nitrates and nitrites under certain conditions of endogenous nitrosation are probably carcinogenic (IARC, 2010).

Faced with this risk to human health, it is therefore important to reduce or eliminate the use of these preservatives in the meat industry. However, this reduction of these compounds is not without consequences. The main risks associated with the reduction or elimination of nitrite in the manufacture of charcuterie products are the reduction of product shelf life, the loss of organoleptic quality and the development of undesirable microorganisms. Aware of these risks, researchers have turned to other alternative methods such as the use of nature preservatives. This is how several works have shown the antibacterial activity of EOs extracted from cinnamon, oregano, rosemary, clove, thyme and pepper (Burt, 2004; Srinivasan, 2007; Tajkarimi et al., 2010; Evrendilek, 2015; Posgay et al., 2022). Moreover, the incorporation of natural antimicrobials in food products also allows to increase the organoleptic characteristics of the products which are appreciated by the consumers (Yu et al., 2021). In this work, the EOs of white *Piper nigrum* of *Penja*, *Thymus vulgaris* and *Syzigiuim aromaticum* were selected to evaluate their effect on the microbiological and sensory quality of sausages during their preservation.

MATERIALS AND METHODS

Vegetal material

The white *P. nigrum* of *Penja* used for the production of EO were purchased from small producers in the locality of *Penja* in the district of *Njombe-Penja* (Littoral, Cameroon). Indeed, the white *P. nigrum* of *Penja* is among the best peppers in the world and is the first product from sub-Saharan Africa to receive a protected geographical indication (PGI) in 2013 (Petchayo et al., 2015). *T. vulgaris* and *S. aromaticum* EOs produced by Renauld et Fils (France) have been purchased.

Microbial material

The strains of *Escherichia coli* ATCC25922, *Salmonella enteritidis* 155A and *Staphylococcus aureus* NCTC10652 were kindly donated by the laboratory of the University of Bologna in Italy.

Extraction of *Penja* EO

Five kilograms of *Penja* white *P. nigrum* were collected to extract the EO. It was carried out by the hydrodistillation method using a Clevenger type apparatus in accordance with International Standards NF ISO 212 and AFNOR (2007).

Determination of MIC of EOs

The MIC of the various EOs was determined by the Macrodilution method. For this, 2 ml of 40 mg/ml EOs stock solutions were introduced into a sterile test tube containing 2 ml of nutritious broth with 2% of glucose. Series of dilutions were performed in order to obtain a range of EO concentrations from 20 to 0.039 mg/ml. Then, 100 µl of each dilution was removed and replaced by 100 µl of bacterial inoculum at a concentration of 10^8 CFU/ml to have a final bacterial concentration of 5.10^6 CFU/ml. Each tube was incubated at 37°C for 24 h and the microbial growth was evaluated using Chloride 2.3.5-triphenyltetrazolium (TTC) (Guinoiseau, 2010). The MIC was the lowest concentration of EO where no microbial growth is visible (Oulkheir et al., 2017).

Antibacterial effect of combined MICs of EOs

The combined effect of MICs of EOs was studied to optimize their effectiveness during sausage preservation. In order to evaluate this antibacterial activity, an inoculum of concentration 5.10^6 CFU/ml for each strain was mixed in a tube with the combined MICs of EOs. The different preparations were then incubated at 37°C for 24 h. After this time, 100 µL of each combination was seeded in Petri dishes containing Chapman medium for *Staphylococcus* NCTC10652, Eosin Methylene Blue Agar (EMB) for *E. coli* ATCC25922 and *S. enteritidis* 155A. The various preparations were incubated at 37°C for 24 h. The combined MICs of EOs have inhibited the maximum of the microbial growth will be retained (CMICR).

Time to kill essay of CMICR

This inhibition test was performed following the method described by Tsuji et al. (2008). In this part of the work, the experimental setup used will be the same as the one used for the antibacterial activity of the EO combination. But in this case, only CMICR will be tested and its antibacterial activity evaluated after 1, 2, 3, 4, 8, 12 and 24 h of incubation.

Effect of CMICR on the growth of microorganisms in sausages

In order to evaluate the antimicrobial power of CMICR in sausages, 5 kg of pork meat (75 lean and 25% fat) were cleaned and then chop using a manual grinder with a 6 mm diameter mesh (Figure 1). Once chopping, 1.5% NaCl was added and the resulting product was then separated into 9 batches of 500 g each. Then, 4 batches were previously treated with CMICR. After this treatment, Batch 1, Batch 2 and Batch 3 were inoculated, respectively with *E. coli* ATCC25922, *S. enteritidis* 155A, and *S. aureus* NCTC10652, each with a concentration of 10^6 CFU/g. The Batch 4 was inoculated with

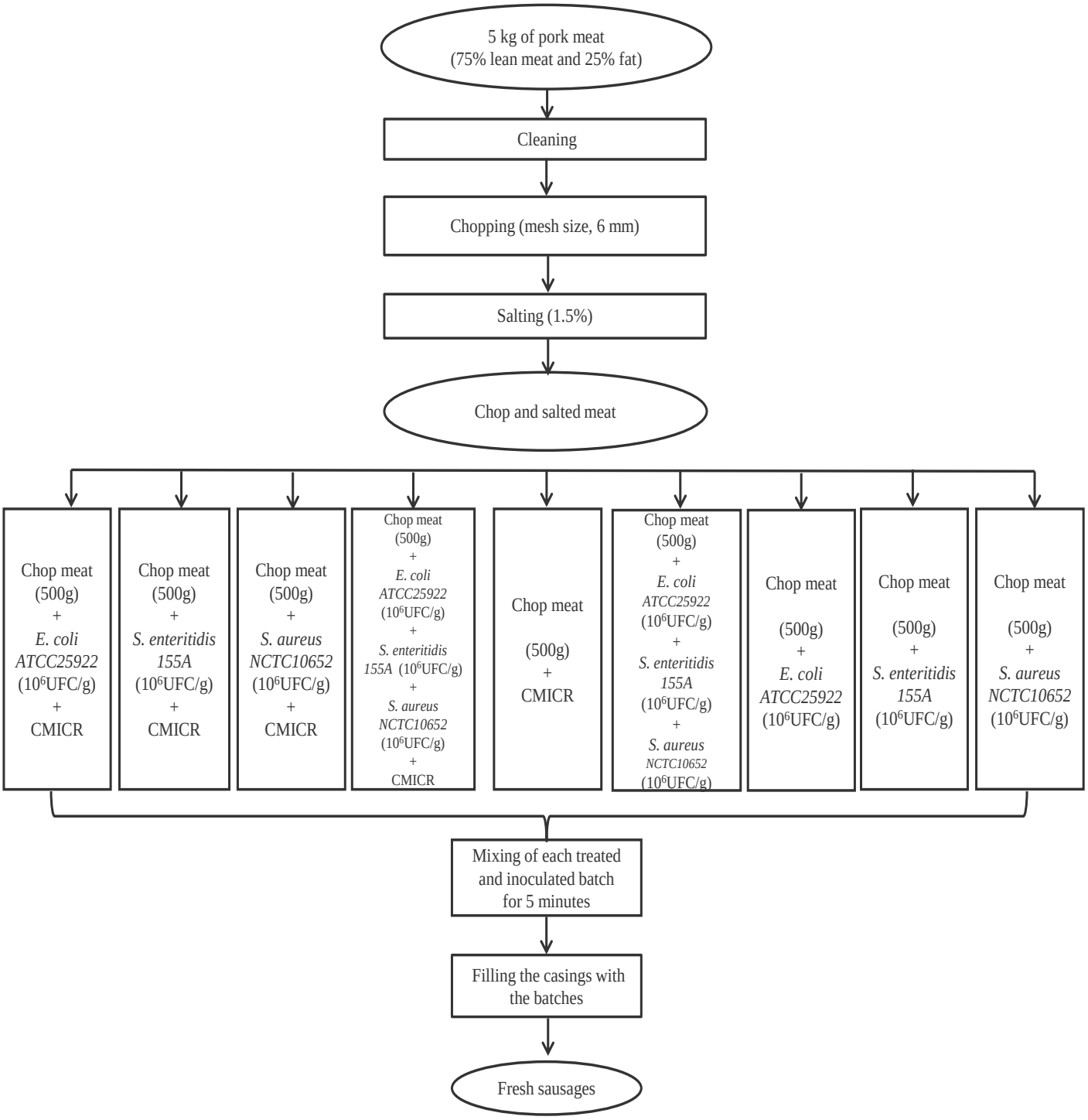

Figure 1. Production, treatment and inoculation of fresh sausages in the laboratory.
Source: Authors

les 03 pathogen bacteria in the same time. The Batch 5, Batch 6, Batch 7 and Batch 8 were inoculated, respectively with *E. coli* ATCC25922, *S. enteritidis* 155A, *S. aureus* NCTC10652, and the combination of the 03 strains served as negative control. Batch 9 was treated only with CMICR and served as a positive control. All sausages were stored at 4°C for 14 days.

Evaluation of microbial growth in the sausages

A mass of 10 g of a randomly sampled sausage was diluted in 90 ml of sterile physiological water (water + NaCl 9%). The sample prepared was homogenized for 3 to 5 min and then a decimal dilution series was carried out. Each dilution was seeded on a Petri

Table 1. Minimum inhibitory concentrations of Eos.

EOs	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella enteritidis*
Thymus vulgaris	625	312.5	1250
Syzigiuim aromaticum	2500	1250	5000
Pipper nigrum of *Penja*	> 20000	> 20000	> 20000

Source: Authors

dish containing EMB media to isolate *E. coli ATCC25922* and *S. enteritidis* 155A and Chapman for *S. aureus* NCTC10652. The Petri dish was incubated at 37°C for 24 h. The Petri dishes containing colonies between 30 and 300 were the choice for bacterial count. Microbial concentrations were obtained using the formula and expressed as CFU/g.

$$C = \frac{N \times Fd}{V}$$

Where C: Microbial concentration expressed in (CFU/g), N: Number of colonies counted on Petri dish, Fd: Dilution factor, and V: Seeded volume (mL).

Sensory analysis of sausages

For the sensory analysis test, the experiment was conducted in three rooms. A room reserved for the preparation of the samples, a room with 05 booths for the tasting and a room reserved for discussion with the panelists. During this analysis, the panelists were asked to provide in a first step their global appreciation for each sausage obtained by attributing a note on a hedonic scale going from 1 to 9 with 1 corresponding to "extremely unpleasant" and 9 to "extremely pleasant". Panelists were also asked to characterize the products by determining the intensity of attributes such as spicy taste, pink coloration, firm texture, spicy smell and flavor of the products on a scale of 1 to 3. The number 1 being "too little", 2 being "Just About Right" and 3 being "too much". To avoid errors, the order of appearance of the attributes was randomized on each sensory analysis form. This analysis also performed a penalty test that identified the various attributes that strongly reduce panelists appreciation of sausages.

Statistical analysis

The averages and standard deviations of the data obtained were calculated using the Excel spreadsheet (Office 2016) and the graphic illustrations were plotted using the Sigma plot 11.0 software. An ANOVA analysis of the sensory test data was performed using XL-Stat software version 2019-3-1 in order to highlight the differences between the panelists assessments.

RESULTS

Determination of the MICs of EOs

Table 1 gives the MICs of the different EOs tested. It can be seen that *T. vulgaris* EO had the lowest MICs compared to the other EOs. It recorded a MIC of 625, 312.5, and 1250 ppm on *S. aureus* NCTC10652, *E. coli* ATCC25922, and *S. enteritidis* 155A respectively. The lowest MIC recorded with *S. aromaticum* EO was 1250 ppm against *E. coli* ATCC25922. However, against all expectations, white *P. nigrum* of *Penja* showed no antimicrobial activity at concentrations equal to 20000 ppm. Despite the lack of activity of the high white *P. nigrum* of *Penja* EO, it will be used in sausages during sensory analysis due to its exceptional organoleptic characteristics sought after worldwide.

Effect of MIC combinations of EOs on microbial growth

Table 2 shows the antibacterial effect of the different combinations of EOs MICs on the growth of *E. coli* ATCC25922 (A), *S. aureus* NCTC10652 (B) and *S. enteritidis* 155A (C). From this analysis, it is apparent that all microorganisms were completely inactivated when tested with the 2CMI and 3CMI combinations. However, the MIC combination rather partially inactivated *S. aureus* NCTC10652 and *E. coli* ATCC25922. In contrast to these microorganisms, the bactericidal effect was observed for *S. enteritidis* 155A for MIC combinations. In view of these results, the combination of MIC of EO of concentration 1250 ppm (*T. vulgaris)* and 5000 ppm (*S. aromaticum*) were retained (CMICR) for further work because of its effectiveness.

Time to kill essay of CMICR

In order to determine the exact time required for the deactivation of microbial growth by CMICR, deactivation kinetics were performed as shown in Figure 2. It was found that after 2 h of exposure with CMICR there was a reduction of *S. aureus* NCTC10652, *E. coli A*TCC25922 and *S. enteritidis 155A* to non-detectable levels.

Antibacterial activity of CMICR in contaminated sausages

The results of the antimicrobial activity of CMICR in sausages contaminated with *E. coli* ATCC25922, *S. aureus* NCTC10652 and *S. enteritidis* 155A are as shown

Table 2. Effect of MICs combinations of EOs on microbial growth.

MIC (ppm) of EO of *Syzicum aromaticum*	**MIC (ppm) of EO of *Thymus vulgaris***		
***Escherichia coli* ATCC25922 (CFU/mL)**	**MIC: 312.5**	**2MIC: 625**	**3MIC: 937.5**
MIC: 1250	5×10	0	0
2 MIC: 2500	0	0	0
3 MIC: 3750	0	0	0
***Staphylococcus aureus* NCTC10652 (CFU/mL)**	**MIC: 625**	**2MIC: 1250**	**3MIC: 875**
MIC: 2500	10^2	0	0
2MIC: 5000	0	0	0
3MIC: 7500	0	0	0
***Salmonella enteritidis* 155A (CFU/mL)**	**MIC: 1250**	**2MIC: 2500**	**3MIC: 3750**
MIC: 5000	0	0	0
2 MIC: 10000	0	0	0
3 MIC: 15000	0	0	0

2MIC: Minimum Inhibitory Concentration multiplied by 2; 3MIC: Minimum Inhibitory Concentration multiplied by 3.
Source: Authors

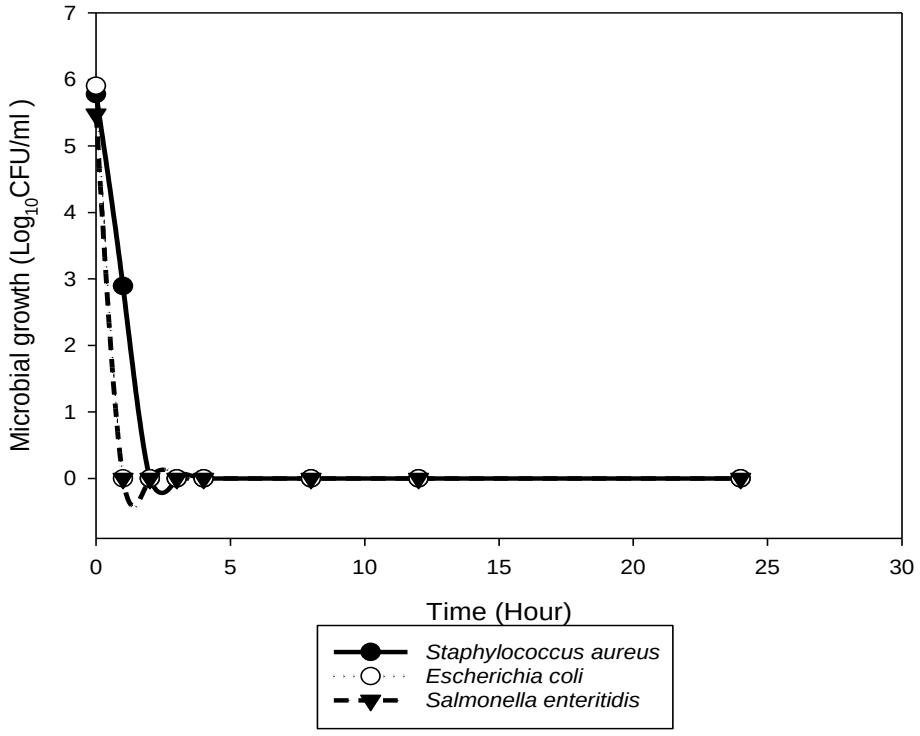

Figure 2. Kinetics of microbial deactivation by CMICR.
Source: Authors

in Figure 3. While the *in vitro* deactivation kinetics showed a total reduction of the microbial load after 2 h, the results obtained in sausages show a gradual and slow reduction of the microorganisms over an average period of 8 days. A reduction in the order of 51, 53 and 44% was recorded for *E. coli* ATCC25922*, S. aureus* NCTC10652 and *S. enteritidis* 155A, respectively after 8, 10 and 14 days of storage before a resumption of growth.

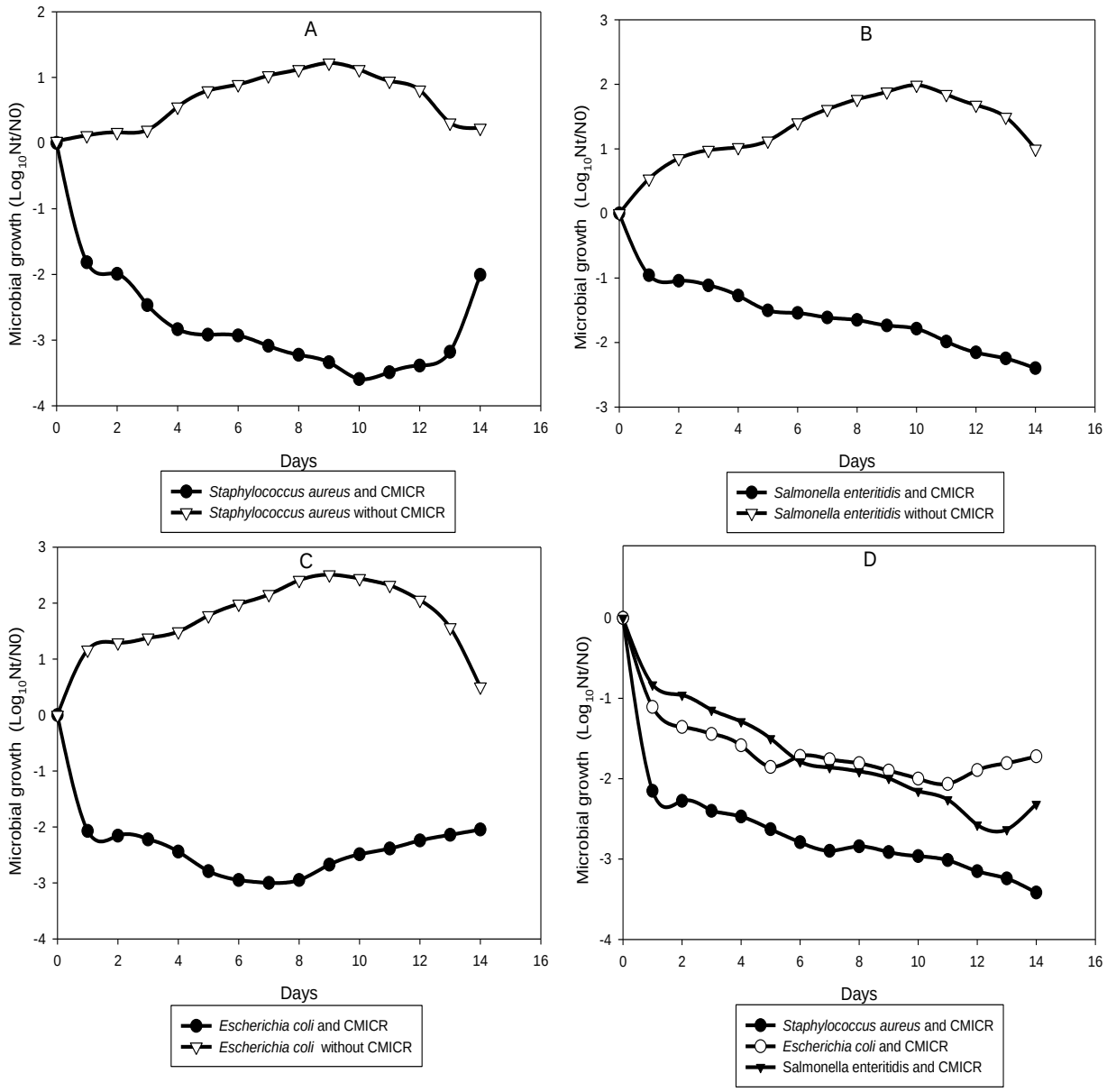

Figure 3. Reduction of *Escherichia coli* ATCC25922 (A), *Salmonella enteritidis* 155A (B), *Staphylococcus aureus* NCTC10652 (C) and combined microorganisms (D) in sausage at 4°C.
Source: Authors

Contrary to the sausages treated with CMICR where a reduction was noted during the conservation, the untreated sausages (controls) saw their average microbial load increase by 16, 33 and 45% for *S. aureus* NCTC10652, *S. enteritidis* 155A and *E. coli* ATCC25922, respectively after 9, 10 and 9 days of conservation before a reduction was observed. In this case, contrary to the sausages contaminated individually by each microorganism, a slight reduction was noted in the sausages contaminated simultaneously by these germs. A reduction of 36, 45 and 50% was recorded in *E. coli* ATCC25922, *S. enteritidis* 155A and *S. aureus* NCTC10652, respectively after 11, 13 and 14 days of conservation before a resumption of growth was observed.

Antibacterial activity of CMICR in naturally contaminated sausages

In this work, the reduction of *S. aureus, E. coli* and *S. enteritidis* naturally present in fresh meat after production was evaluated (Figure 4). Compared to the results observed in the sausages contaminated in the laboratory

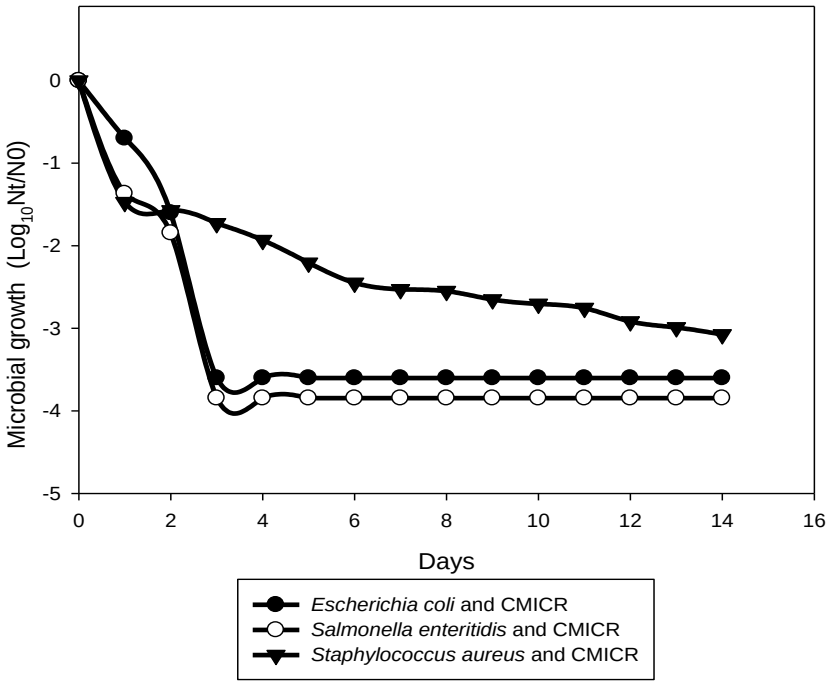

Figure 4. Reduction of microbial growth by CMICR in naturally contaminated sausages at 4°C.
Source: Authors

by microorganisms, we note a reduction of the concentration of *E. coli* and *S. enteritidis* to a non-detectable threshold in the sausages after the 3 days of conservation at 4°C. In contrast, *S. aureus* was reduced by only 48% after 14 days of storage at 4°C. This result shows that the degree of effectiveness of CMICR in deactivating *E. coli* and *S. enteritidis* at non-detectable levels is highly dependent on their concentration in the sausages.

Overall appreciation of the sausages

The sensory analysis was carried out with the sausages produced with different EOs used in this work. A total of 03 sausages were obtained. We have sausages treated with EOs of white *P. nigrum* of *Penja* (SAUS 324), sausages treated with EO of *P. nigrum* of *Penja* associated with the CMICR (SAUS 589) and sausages treated with the CMICR (SAUS 912). Not treated sausages with EOs (SAUS 102) and commercial sausages (SAUS 706) were also subjected to sensory analysis. The results show that sausage SAUS 324 and SAUS 589 were appreciated (score 6) by panelist on par with the commercial sausage (SAUS 706). The sausages SAUS 912 and SAUS 102 were less appreciated (score 5). The sausages SAUS 324 and SAUS 589 that were appreciated by the panelists are those in which white *P. nigrum* of *Penja* was part of the seasoning. Table 3 shows the overall acceptability of the different sausages.

Characteristics of the sausages

The results of the JAR test (Figure 5), which highlighted the specific characteristics of each sausage, showed that sausage SAUS 324 was judged to have a good spicy taste (67%), good spicy smell (61%), good salty flavor (72%) and good firm texture (50%). For the sausage SAUS 539, panelists characterized it as too much spicy taste (56%), too much spicy smell (50%), too much salty flavor (56%), too little firm texture (50%) and too little pink color (61%). The sausage SAUS 912 which was less appreciated compared to sausage SAUS 324 and SAUS 589 has a good spicy taste (44%), good spicy smell (50%), good salty flavor (50%), but with too little firm texture (61%) and too little pink color (89%). The sausage SAUS 102 is characterized by a good taste (56%), too little spicy smell (78%), too little salty flavor (50%), too little firm texture (61%) and too little pink color (67%).

Penalty test of the sausages

During this analysis, the attributes taken into account are those whose penalties have a threshold of 20% and which lead to an increase in the average (>1). It appears

Table 3. Overall acceptability of different sausages.

Sausages code	Composition of the sausages	Overall assessment	Significance	
			F	Pr > F
SAUS 706	Commercial sausage	6 ± 1.63		
SAUS 324	Sausages with *Piper nigrum* of Penja	6 ± 1.63		
SAUS 589	Sausages with *Piper nigrum* of *Penja* + CMICR	6 ± 1.54	0.88	0.48
SAUS 912	Sausages + CMICR	5 ± 1.46		
SAUS 102	Sausage without CMICR and *Piper nigrum* of *Penja*	5 ± 1.81		

Hedonic scale: 1= Extremely unpleasant; 2=Very unpleasant; 3= Enough unpleasant; 4=Unpleasant; 5=Not unpleasant - Not pleasant; 6= Enough pleasant; 7=Pleasant; 8=Very pleasant; 9=Extremely pleasant.
Source: Authors

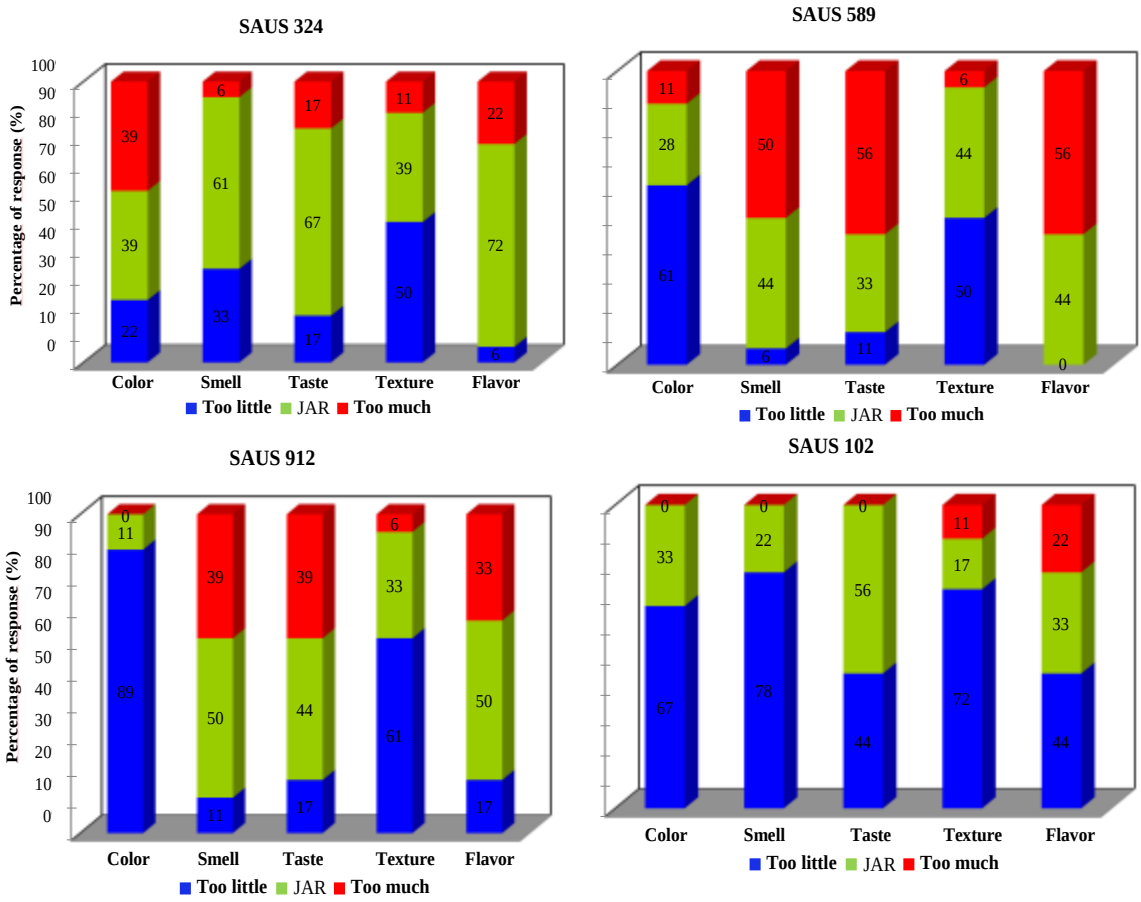

Figure 5. Characteristics of the different sausages produced.
Source: Authors

from this test that the too little spicy smell and the too little pink color of sausage SAUS 324 significantly reduce its acceptability. While too much salty flavor, too little pink color and too much spicy smell were penalizing sausage SAUS 589. Taste and too little spicy smell were the 02 characteristics that reduced the acceptability of sausage SAUS 912. The results of the penalty analysis of the sausages are as shown in Figure 6.

DISCUSSION

In this work, the EOs of white *P. nigrum* of *Penja* did not show any antimicrobial activity on the main pathogens

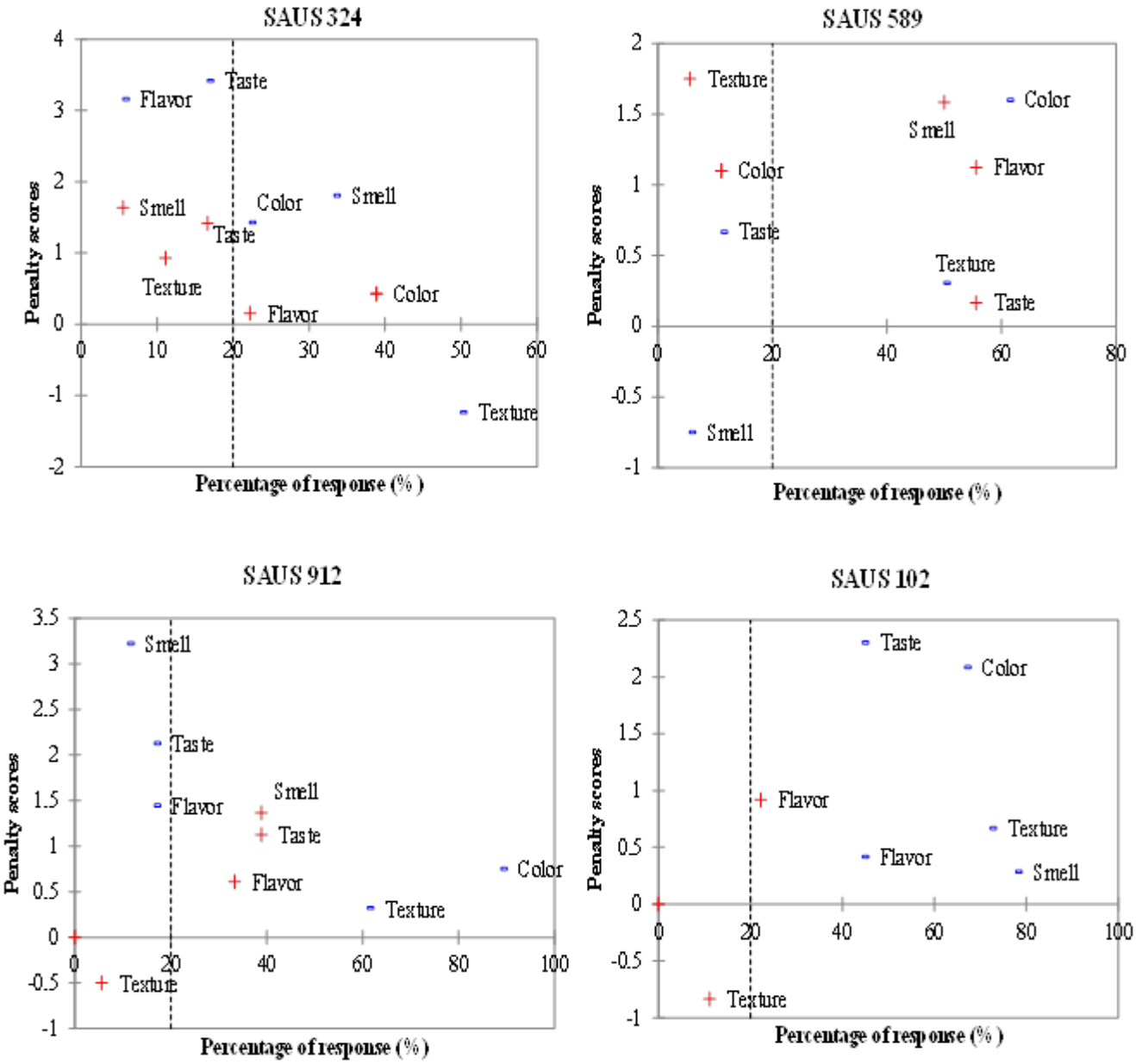

Figure 6. Results of the penalty analysis of the different sausages.
Source: Authors

studied. This result is contrary to some works that showed the black *P. nigrum* would contain substances such as piperine which is responsible for its antimicrobial power. The work of Ojo et al. (2021) showed a great antibacterial power of purified piperine on *S. aureus* and *E. coli* and *S. enterica*. However, the work done by Arsana et al. (2022) showed that white *P. nigrum* would also contain Piperine. This difference observed in the antimicrobial activity could be explained by the difference in the composition of the different *P. nigrum* tested. Indeed, the antimicrobial activity of an EO is closely related to its chemical composition which itself also depends on the species, subspecies or variety of the plant used, the geographical area and the period of harvest, the method of drying or extraction of the EO (Moraru et al., 2019). In contrast, EOs of *T. vulgaris* and *S. aromaticum* were found to be effective against *E. coli* ATCC25922, *S. enteritidis* 155A and *S. aureus* NCTC10652. This antimicrobial activity is thought to be due to the presence of numerous oxygenated terpenoid derivatives in the composition of these different EOs (Angane et al., 2022) and the higher percentage of phenolic compounds in EO higher its antimicrobial properties (Boskovic et al., 2015). The presence of

thymol and carvacrol in *T. vulgaris* EO and eugenol in *S. aromaticum* EO as the predominant compound (Ramsey et al., 2020) could also explain their high activity. These antibacterial compounds mainly act on microbial cells by destabilizing the cell architecture, leading to the increase of membrane permeability and the disruption of many cellular activities such as energy production, membrane transport and other metabolic functions (Di pasqua et al., 2006; Devi et al., 2010; Swamy et al., 2016).

Contrary to the MICs of the EOs obtained, the CMICR resulted in a significant reduction in the growth of *E. coli* ATCC25922, *S. enteritidis* 155A and *S. aureus* NCTC 10652. This significant reduction is thought to be related to the combined action of the antimicrobial components of *T. vulgaris* and *S. aromaticum* EOs acting synergistically or additively to induce a plethora of antibacterial effects (Bassolé and Juliani, 2012). Some works have shown that mixing pure compounds of Thymol and Eugenol could increase their antibacterial effect by 50% on *E. coli* and *Bacillus cereus* (Gallucci et al., 2009, Pei et al., 2009). Das et al. (2022) also stated that the EOs reduced the microbial growth and increased the shelf life of chicken meat during refrigeration at 4°C.

In the determination of the deactivation kinetics, the CMICR inhibited at a non-detectable threshold the growth of *E. coli* ATCC25922, *S. enteritidis* 155A and *S. aureus* NCTC10652 after 2 h. This rapid reduction could be explained by the contact in liquid medium of the tested germs with a diversity and high concentration of antimicrobial compounds from the combined EOs. The increase in these different parameters would have led to a series of reactions among which the increase in membrane permeability and the destruction of cell membranes (Nazzaro et al., 2013).

While *in vitro* deactivation kinetics showed deactivation at non-detectable levels of microbial growth after 2 h, in sausages contaminated with *E. coli* ATCC25922, *S. enteritidis* 155A and *S. aureus* NCTC10652 and stored at 4°C, a gradual and partial deactivation of the microbial load was observed up to the 14th day. This longer deactivation time could be explained to be the reduction of the effectiveness of CMICR due to the interaction of its compounds with the constituents of the food matrix such as lipids, proteins and ingredients present (Da Silva et al., 2021). In addition, due to their lipophilic nature, EOs are dissolved in the oil phase of foods, making them less available to act against bacteria present in the water phase (Mejlholm and Dalgaard, 2002). The work done by Tassou et al. (1995) on fish pate and egg salad showed a reduction in the antibacterial activity of mint oil on *Listeria monocytogenes* and *S. enteritidis* mainly due to the high lipid content of these foods. Similarly, Pol et al. (2001) demonstrated that the high concentrations of protein in the milk act as a limiting factor in the antibacterial activity of Carvacrol against *B. cereus.*

The growth of *E. coli* ATCC25922 and *S. aureus* NCTC10652 observed on day 8 and 10, respectively during storage in sausages treated with CMICR is thought to be related to the instability of EOs over time due to their volatility. To this, it can be added a highly nutritious environment of the food matrix and can therefore increase the recovery rate of microorganisms that have undergone some type of stress (Gill et al., 2002).

The evaluation of the antimicrobial activity of substances in laboratory conditions is associated with specific standard microbial concentrations to define efficacy parameters (MIC and MBC). These microbial concentrations are sometimes different from those naturally encountered in food. In this work, it was noted that meat taken under real conditions of sale had a lower microbial concentration than contaminated meat in the laboratory. In the efficacy tests of CMICR on naturally contaminated meat, a total reduction of the microbial level was observed after 3 days of storage at 4°C. The antimicrobial activity of CMICR would thus depend on the microbial concentration present in the treated sausages. According to the work of Burt (2004), the effectiveness of antibacterial substances is significantly influenced by the microbial concentration of food.

While sanitary quality is an important factor in consumer choice of sausages, organoleptic characteristics also remain one of the major factors contributing to their acceptability. Among the sausages produced, only sausage SAUS 324 and SAUS 589 were appreciated in the same way as the commercial sausage (SAUS 706). We note that these two sausages have in common the presence of *P. nigrum* of *Penja* in their composition. This would explain the appreciation of these sausages by the panelists. Indeed, *P. nigrum* cultivated near the *Penja* locality in Cameroon is considered one of the best peppers in the world. *P. nigrum* of *Penja* is the first sub-Saharan African product to obtain a Protected Geographical Indication (PGI) due to its exceptional aroma and flavor (Folefack et al., 2022). Although appreciated for some of their attributes, SAUS 324 and SAUS 589 sausages have been unfortunately depreciated for their small pink color which is explained by the absence of nitrite and nitrate during their preparation. The nitrite used in the production of commercial sausages, through different chemical reactions, leads to the formation of nitric oxide (NO) which will bind to myoglobin to form nitrosomyoglobin, a stable and red compound, responsible for the typical color of sausages (Honikel, 2008).

Conclusion

At the end of this work, it was found that CMICR eliminates the growth of *E. coli* ATCC 25922 and *S. enteritidis* 155A in naturally contaminated sausages at a non-detectable level after 3 days of storage. The sensory analyses show that all the sausages treated with EO of

Penja P.nigrum in this case sausage SAUS 324 and SAUS 589 were appreciated by the consumers. But the too small pink color of these sausages must be improved in order to increase their appreciation by consumers. However, in order to ensure consumer safety and appreciation of this product, CMICR and EO *P. nigrum* of *Penja* must be mixed during sausage production.

CONFLICT OF INTERESTS

The authors have not declared any of conflict of interests.

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Prevalence of rotavirus group A and associated genotypes detected in calves in Southern Highlands and Eastern Tanzania

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Rotavirus group A (RVA) causes neonatal calves diarrhoea, causing massive economic losses in many countries due to deaths, treatment costs, and stunted growth. However, little is known about Tanzanian cattle's RVA burden and genotypes. The prevalence and genetic diversity of rotaviruses circulating in cattle in Southern and Eastern Tanzania regions were investigated. A total of 272 faecal samples collected from calves (102 from Mbeya, 89 from Iringa, and 81 from Morogoro) were tested for RVA. 30 (11.03%) of the 272 samples tested positive for RVA, 7 (6.8%) of the 30 RVA positive samples were from Mbeya, 23 (28.39%) from Morogoro, and no positive samples were found in the Iringa region. Using RT-PCR, 20 samples were identified as G10 genotypes, while the remaining 10 samples were untypeable with the primers used, whereas all P genotypes were untypeable. To confirm the RT-PCR results, representative samples were chosen and sequenced in the ABI 3130XL using a Big-dye Terminator kit with Sanger dideoxy sequencing method. Phylogenetic analysis and nucleotide comparison revealed that G10 was closely related to and clustered with human G10 strains from neighboring Kenya, implying that circulating G10 strains may have the potential for zoonotic transmission. When circulating G10 amino acid alignment was compared to bovine vaccine strain B223, our G10 samples had various nucleotide substitutions at antigenic epitopes, which resulted in the acquisition of glycosylation sites suggesting that G10 has the potential to evade neutralization antibodies induced by a vaccine. Our study provided preliminary data on RVA in cattle in Tanzania, paving the way for further research into the rotavirus epidemiology and risk factors associated with RVA in cattle in Tanzania.

Key words: Cattle, rotavirus groups A, G10 genotype, Tanzania.

INTRODUCTION

Rotavirus group A (RVA) is the causative agent of diarrhea-related cases and mortality in children under the age of five and young animals worldwide (Liu et al., 2012; Komoto et al., 2016; Troeger et al., 2018). The RVA has been found in a variety of animal species, including cattle, pigs, dogs, horses and other species (Martella et al., 2010: Li et al., 2016; Papp et al., 2013). In cattle, responsible for neonatal calves' diarrhea, causing massive economic loss in many countries due to deaths, treatment costs, and stunted growth (Bellinzoni et al., 1989; Garaicoechea et al., 2006; Bassera et al., 2010). Some countries have implemented vaccine programs to combat rotavirus infection in cattle due to its significant economic impact (Rodrguez-Limas et al., 2009; Swiatek et al., 2010). However, there is no RVA vaccination program in place in Tanzanian cattle, despite the fact that it has been effectively implemented in children under five since 2013 (Moyo et al., 2011). A few studies have examined the prevalence of RVA in African nations. These studies found that the RVA prevalence was 13.1% in the Ivory Coast (Yahaya et al., 2018), 3.64% in Ethiopia (Debelo et al., 2021), 1.8% in Nigeria (Garba et al., 2020) and elsewhere, 8.8% in Korea (Chae et al., 2021), as well as 5.11% in Bangladesh (Barua et al., 2019). In Tanzania, a cattle is one of the major source of protein and a significant source of income. The cattle population is rapidly growing; it was estimated to be 19 million cattle in 2010 and has recently increased to 28 million cattle by 2020 (Statista, 2022). Despite the significant contribution of cattle to the national economy, little is known about the burden, prevalence and genotypes of RVA in cattle in Tanzania. Because of the potential impact of rotavirus in cattle, understanding the burden and genotypes circulating in cattle is critical in protecting cattle health and thus improving human livelihood.

Several RVA strains are zoonotic and have been linked to a number of diarrhea cases and deaths in children under the age of five (Pecenka et al., 2017; Troeger et al., 2018). In Bangladesh, a bovine-like RVA was discovered in children suffering from diarrhea (Afrad et al., 2013). Because humans and animals coexist in Tanzania, there is a risk of zoonotic transmission to humans. Zoonotic transmission is known to drive rotavirus diversity, which may have implications for vaccine performance (Doro et al., 2015; Vlasova et al., 2017). As a result, highlighting bovine strain profiles and investigating their zoonotic potential is critical in examining the design of strategic controls for RVA intervention in both humans and animals.

The RVA virus is a double-stranded RNA virus in the Reoviridae family. Its genome is made up of 11 segments, six of which code for structural proteins (VP1-VP4, VP6 and VP7) and five of which code for non-structural proteins (NSP1-NSP5/6) (Estes and Kapikian, 2007). The VP4 and VP7 proteins are the most important because they form a dual classification system (VP7 forms G1.....Gnth genotypes and VP4 forms P[1],P[2].....P[nth] genotypes) and are used for eliciting independent immune responses, making them crucial in vaccine development (Matthijnssens et al., 2008).

In humans and animals, 41 G genotypes and 57 P genotypes have been identified so far (RCWG, 2022). The most common G genotypes found in cattle are G6, G10, and G8, while the most common P genotypes are P [5], P[11], and P[1] worldwide. Other genotypes detected sporadically include G types G1-G3, G5, G11, G15, G17, G21, and G24) and 11 P types P[3], P[6], P[7], P[14], P[17], P[21], P[29], and P[33], with G6P[5], G6P[11], and G10P[11] being the most common (Papp et al., 2013).

Because of RVA infection in cattle reported to have economic drawbacks and limited studies on the RVA circulating in cattle in Tanzania, this study was carried out to better understand the RVA burden and genetic relationship of rotavirus genotypes circulating in cattle in Tanzania. The study was conducted in a selected region with a large cattle population and animals-human interaction found in the Southern highland (Mbeya and Iringa) and Eastern (Morogoro) Tanzania.

MATERIALS AND METHODS

Description of the study area and design

This cross-sectional study was carried out in three Tanzanian districts, namely Mbarali, Kilolo, and Mvomero, which were chosen for their large cattle populations and animal-human interaction from three regions, namely Mbeya, Iringa (Southern highlands), and Morogoro (eastern Tanzania). Between April 2019 and May 2020, samples were collected. Mbarali is located between latitudes S07" 41' and S09" 25' and longitudes E33" 40' and E35" 40' above sea level, at an elevation of 1000 to 1800 meters (masl). The cattle population was estimated to be 198,316 (HWMB, 2020). In the Iringa region, the Kilolo district is located between latitudes 35" 54' E and 35" 57' E and longitudes 8" 01' S and 8" 06' S. there are 79500 cattle (HWKI, 2020). Mvomero is situated between 6'07' and 7'05' South and 37'17' and 37'65' east. The district altitude ranges from 300 to 400 masl in Turiani, Mvomero, and Mzumbe divisions to 1000 to 1700 masl in Mgeta division. The cattle population was estimated to be 125988 (HWMV, 2020).

Ethical considerations

The permit on the subject of animals was obtained from Sokoine University of Agriculture via the CVMBS College Research

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Innovations and Publication Committee (SUA/CVMBS/R.1/2019/1). Permission was also granted by the Regional Administrative Secretary (RAS), District Administrative Secretary (DAS).

Sample collection

The aseptic collection of feces was done on calves younger than six months. Directly taken sample from the rectum of chosen calves, either with or without diarrhea. Approximately a pea-size feacal sample was picked from the animal and placed in duplicate into sterile cryogenic vials (Corning, Lowell, USA) and stored in liquid nitrogen. The vials contained 1 ml Trizol reagent (Life Technologies Corporation, Carlsbad, USA) and 1 ml of viral transporting media (Remel Micro test M6, Lenexa, USA). Samples transported in liquid nitrogen to Sokoine University of Agriculture. On arrival, samples were stored in a -80 ^{0} C freezer (NUAIRE, Plymouth, USA).

Sample preparation and RNA extraction

Samples were removed from the ultralow temperature freezer and placed in a class II biological safety cabinet (SterilGard, Sanford, USA) allowing them to thaw. Afterward, samples were centrifuged at 5000 g for 8 min using Centrifuge 5717R (Eppendorf, Hamburg, Germany). Then 250 µL of supernatant was harvested and used for RNA ex-traction using direct zol-RNA MiniPrep Kit (Direct zol RNA MiniPrep, Tustin, United States of America) following the manufacturer's instructions. The final volume of dsRNA after extraction was 50 µL, 25 µL of the total volume was aliquoted into another PCR tubes and stored in -80^{0} C ultra-freezers and one was used for cDNA synthesis.

cDNA synthesis

The dsRNA was first denatured at 97^{0} C for 5 min and chilled on ice for 2 min, 8 µl of the denatured RNA was used for cDNA synthesis using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, USA) manufacturer's instructions. The final volume of the cDNA was 20 µl which was divided into two aliquots of 10 µl and stored at -80 ^{0} C.

Detection of Rotavirus group A

Rotavirus group A (RVA) was detected using primers (Macrogen, Seoul, Republic of South Korea) NSP3-F and NSP3-R yielding 87 bp on gel electrophoresis as described previous (Amimo et al., 2015). The cDNA obtained was used to detect RVA using the Invitrogen Platinum Taq kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions.

Briefly, the PCR master mix had 2.5 µL 10X PCR Buffer, 0.75 µL 50 mM MgCl2, 0.5 µL 10 mM dNTP, 0.1 µL Platinum Taq DNA polymerase, 1 µL 10 µm NSP3 forward primer, 1 µL 10 µm NSP3 reverse primer and 18.15 µL molecular grade water. Then 1µL of DNA template, RNA extraction control, positive control (Rotarix Vaccine and Bovine rota-virus Indiana strain) and PCR control (molecular grade water) as negative control were added to the respective PCR master mix. The Gel electrophoresis was performed on the PCR product using 3% agarose gel (AMRESCO, Solon, USA) stained with gel red nucleic acid stain (Biotium, Fremont, USA) at 110 voltage for 1.30 hr. The DNA marker (Bionexus, Oakland, USA) of 15 µL was loaded along with the PCR product. The gel visualization of amplicon size was performed using Dual UV Trans-illuminator (Analytic Jena US, Upland, USA).

Genotyping of Rotavirus group A

The G and P genotyping was performed by multiplex reverse Transcriptase nested PCR using primer sets and amplification conditions as previously described (Ansari et al., 2013). The Invitrogen Platinum Taq kits were used for PCR amplification following manufacture instructions. The primer set (VP7-F and VP7-R) for round 1 PCR target the VP7 gene for all RVA resulting in a band size of 881 bp on gel electrophoresis. The primer sets for round 2, multiplex PCR for G typing targeted G1, G2, G3, G4, G8, G9, G10 and G12 Whereas for the P typing, the primer set (Con3 and Con2) (Ansari et al., 2013) for round 1 targeted the VP4 gene for RVA which produces a band size of 876 bp on gel electrophoresis. The round 2 targeted P[4], P[6], P[8], P[9], P[10] and P[11] (Ansari et al., 2013). Gel electrophoresis was performed using a 2% agarose gel stained with gel red at 110 voltages for 1.30 hr. The gel visualization of amplicon size was done using a gel documentation system.

Sequencing of VP7 gene

To confirm RT-PCR results and for determination of genetic and antigenic relationship of the RVA genotypes, the PCR product for round one RT-PCR for VP7 were sent for sequencing at the Macrogen in Netherland. The PCR product were sequenced using Sanger dideoxy sequencing method on ABI 3130XL machine (Applied Biosystems, Carlsbad, USA) using a BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA). The same primer used in round 1 was used for sequencing.

Sequence processing

The ABI sequence files of the forward and the reverse sequences were edited aligned and consensus sequences generated using the BioEdit sequence alignment Editor 7.2.5. The rotavirus genotypes were assigned based on the nucleotide percentage identities using the ViPR (ViPR, 2021) based on the RotaC algorithm developed by Maes et al. (2009) and BLAST. The cutoff value for genotypes assignment was 82% nucleotides identities for both VP7 and VP4 typing (Matthijnssens et al., 2008).

Phylogenetic analysis and amino acid alignments

Using MEGA X, the best fit substitution model was identified based on the lowest BIC scores (Bayesi-an information Criterion). Tamura 3-parameters T92 (BIC 2695) models were found to best fit the data (Nei and Kumar, 2000; Kumar et al., 2018). Therefore, the phylogenic tree was constructed using the Maximum likelihood method and the Tamura 3-parameters with 1000 bootstrap replicates (Kumar et al., 2018; Tamura, 1992). The reference sequences for the analysis were found by blasting our study sequence nucleotides Blast and the translation done by blastx on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers for strains representing lineages were obtained from previous publications and nucleotide retrieval was performed using nucleotide blast. The alignment of deduced amino acid was performed using BioEdit sequence alignment Editor 7.2.5.

RESULTS AND DISCUSSION

Prevalence and genotypes distribution of RVA in calves

A total of 272 fecal samples (102 from Mbeya, 89 from

Table 1. Site infection rate of RVA in calves.

Region	District	Ward	Village	N=272	Rotavirus positivity rate (%)
Mbeya	Mbarali	Mawindi	Itipingi	9	4 (44.44)
			Kangaga	9	1 (11.11)
			Manienga	8	0
		Chimala	Igumbilo	5	0
			Mwale	9	0
		Ihai	Ihai	14	1 (7.14)
			Kibaoni	2	0
		Luhanga	Luhanga	46	1 (2.17)
Morogoro	Mvomero	Mhondo	Kichangani	6	5 (83.33)
		Manyinga	Diongoya	8	3 (37.5)
		Mtibwa	Lukenge	20	8 (40)
		Melela	Kibaoni	26	4 (15.38)
		Mangai	Malandizi	21	3 (14.25)
Iringa	Kilolo	Ihimbo	Utengule	9	0
			Itarula	11	0
			Ihimbo	13	0
		Ng'uruwe	Lukani	12	0
			Ng'uruwe	11	0
		Image	Image 8	11	0
			Uhominyi	6	0
			Ilawa	7	0

Source: Authors

Iringa, and 81 from Morogoro) collected from calves were tested for RVA, with 30 (11.03%) samples showing a band size of 87 bp on gel electrophoresis. The 7 (6.8%) of the 30 RVA positive samples were from Mbeya, 23 (28.39%) from Morogoro, and no positive samples were found in the Iringa region. The RVA positivity rate for the sites was shown in Table 1. The results show that the infection rate varies by region, with lower prevalence in Mbeya and Iringa than in Morogoro possibly due to sample collection criteria of testing symptomatic and asymptomatic calves. The results were comparable to the prevalence of RVA reported in other African countries such as Ivory Coast (Yahaya et al., 2018), Tunisia (Fodha et al., 2005), Nigeria (Babalola et al., 20200. But also lower prevalence compared to this study have been reported in Nigeria (Garba et al., 2020), Ethiopia (Debelo et al., 2021). Also the results were comparable to other countries outside Africa such as Australia (Swiatek et al., 2010), India and Mexico (Rodrguez-Limasa et al., 2009; Basera et al., 2010). However, six European countries have reported a higher prevalence of RVA in cattle (Midgley et al., 2012). The prevalence could not be compared to other neighboring countries due to the lack of information on RVA infection in cattle from other East African countries. Infection with rotavirus in cattle was first documented in this study. Bovine rotavirus diarrhea is a high morbidity condition that costs cattle herders significantly in terms of treatment expenses and decreased weight gain in infected animals (Bartels et al., 2010). This study offered crucial details about the prevalence of rotavirus infection in cattle in Tanzania. Unwrap for additional research on the prevalence of RVA in cattle and the risk factors that are linked to it. The study did have some limitations, though; it was unable to identify any additional pathogens that might be responsible for diarrhea. This may aid in locating the pathogens responsible for the diarrhoea in Tanzanian cattle. Additionally, only a small number of positive samples were found, making it impossible to link the presence or absence of diarrhea in calves with RVA in those animals.

Due to the failure of other samples to amplify in rounds one and two of G and P typing RT-PCR, only two samples were able to be sequenced for VP7 and none for VP4. These untypeable G and P may not have amplified in round one of P typing RT-PCR and some G typing RT-PCR due to primer failure caused by mutation or insufficient nucleic acid materials (Gómara et al., 2001; 2004). Only the G10 genotype was found in cattle circulating in Mbarali and Mvomero, along with untypeable

P genotypes. The G10 genotype has been identified as one of the most common genotypes circulating in cattle in several African countries, including Ivory Coast, Morocco, and Nigeria (Monney et al., 2019; Babalola et al., 2020; Amine et al., 2020). The G10 reported has been linked to numerous cases of diarrhea in calves (Badaracco et al., 2013; Monney et al., 2019). The G10 is the second-most common genotype worldwide, accounting for 20% of the circulating genotypes in calves (Papp et al., 2013). However, they also make up 20% of the circulating genotypes in many different countries (Castells et al., 2020). It has frequently been reported in combination with P [11] (Medeiros et al., 2019; Hossain et al., 2020; Castells et al., 2020), however, P[11] was not identified in this study. Since G10 genotype is common and has a significant impact on health in many nations, some vaccines to treat calf diarrhea now contain G10 in their formulation. For instance, the BOVILIS® GUARDIAN® (GUARDIAN®, Merck Animal Health, USA) vaccine's cocktail includes the rotavirus strains G10 and G6 as well as types 1 and 3 of coronavirus, types C and D of Clostridium perfringens, and type K99 of Escherichia coli (Papp et al., 2013; Karayel et al., 2017). Primers were used in this study to genotype P [4], P[6, P[8, P[9], P[10], and P[11] P genotypes. Other P genotypes, such as [P6], P [14], and P [15], have been reported in conjunction with G10 (Hossain et al., 2020; Babalola et al., 2020). The P genotypes were all untypeable, which could be due to primer failure due to mutation that why could not amply in round one of P genotyping RT-PCR and only 2 samples yield a PCR product for round one and two of G genotyping RT-PCR or different strain not recognized by used primer (Gómara et al., 2001; 2004). The study was unable to obtain sequence data for the corresponding P genotype, and as a result, it was unable to fully document the genetic diversity of G10.

The findings provide preliminary evidence of G10 genotype for the first time in Tanzania; therefore, additional research is required to identify other rotavirus genotypes in cattle in Tanzania for better development of control programs including vaccination. The sequences of the G10 genotypes have been deposited in the gene bank and assigned the accession numbers MW718929 and MW718930.

Phylogenetic analysis VP7 gene

The bovine strains were phylogenetically related (97%) and belonged to lineage II. The authors Bovine G10 clustered with G10 strains isolated from humans in neighboring Kenya (Figure 1), with nucleotide identities ranging from 97.13% to 97.89% shared suggesting their potential of G10 genotypes for zoonotic transmission. In symptomatic African children and elsewhere, a number of bovine-human G10 and human-bovine-like reassortant strains have been identified (Esona et al., 2011). However, more research into the genomic properties of these genotypes is required to understand the possibility of reassortment events and their origin. Interspecies and reassortment events are known to contribute to the variety of rotavirus strains that affect humans (Nakagomi and Nakagomi, 2002; Gomara et al., 2004; Martella et al., 2006; Rajendran et al., 2014) in which higher strain diversity may have an impact on vaccine effectiveness. Understanding these events is therefore critical in designing effective strategic control programs. Other African countries that have reported G10 genotypes in cattle include South Africa, Mozambique, and Ivory Coast (Monney et al., 2019; Strydom et al., 2021), but the majority of their G10 strains are from lineage V (Strydom et al., 2021). Our strain clustered distantly from vaccine strain B223 in lineage IV, implying that the circulating strains G10 is may be antigenically distinct.

Amino acid analysis

Amino acid substitution was discovered at the antigenic region of the circulating G10 strain compared to the bovine vaccine strain B223 on amino acid alignment (Table 2). The antigenic region variation has been linked to the ability of the wild type strain to evade neutralization antibody induced by the vaccine strain (Hoshino et al., 2004; Aoki et al., 2009; Trinh et al., 2007; Banyai et al., 2009; Harastani et al., 2020). However, more research on analysis is required to include a large number of G10 strains from across the country in order to make a conclusive observation. In our strain RVA/Cow-wt/TZA/Morogoro-RC017/2019/G10P[x], the amino acid asparagine (N) at position 96 was replaced by serine (S), resulting in the acquisition of glycosylation site at 94 (NDS). Other glycosylation sites at 69 (NAT) were found to be conserved in bovine vaccine strain B223. Similar findings were reported in Ghanian G10 strains (Badaracco et al., 2013). The glycosylation of amino acid residues has been reported to increase virus pathogenicity and resistance to monoclonal antibody by covering the virus with polysaccharide thus hiding the protein part of the virus (Caust et al., 1987; Ciarlet et al., 1994; 1997).

Conclusion

For the first time, rotavirus infection in Tanzanian cattle was reported in the study; therefore, there is a need for ongoing monitoring of rotavirus infection in cattle, as well as for awareness raising and an evaluation of the financial costs and potential effects on public health. Our bovine genotypes clustered with a human strain from a neighboring country, indicating the possibility of zoonotic transmission to human; however, further research into their genome properties is required to understand

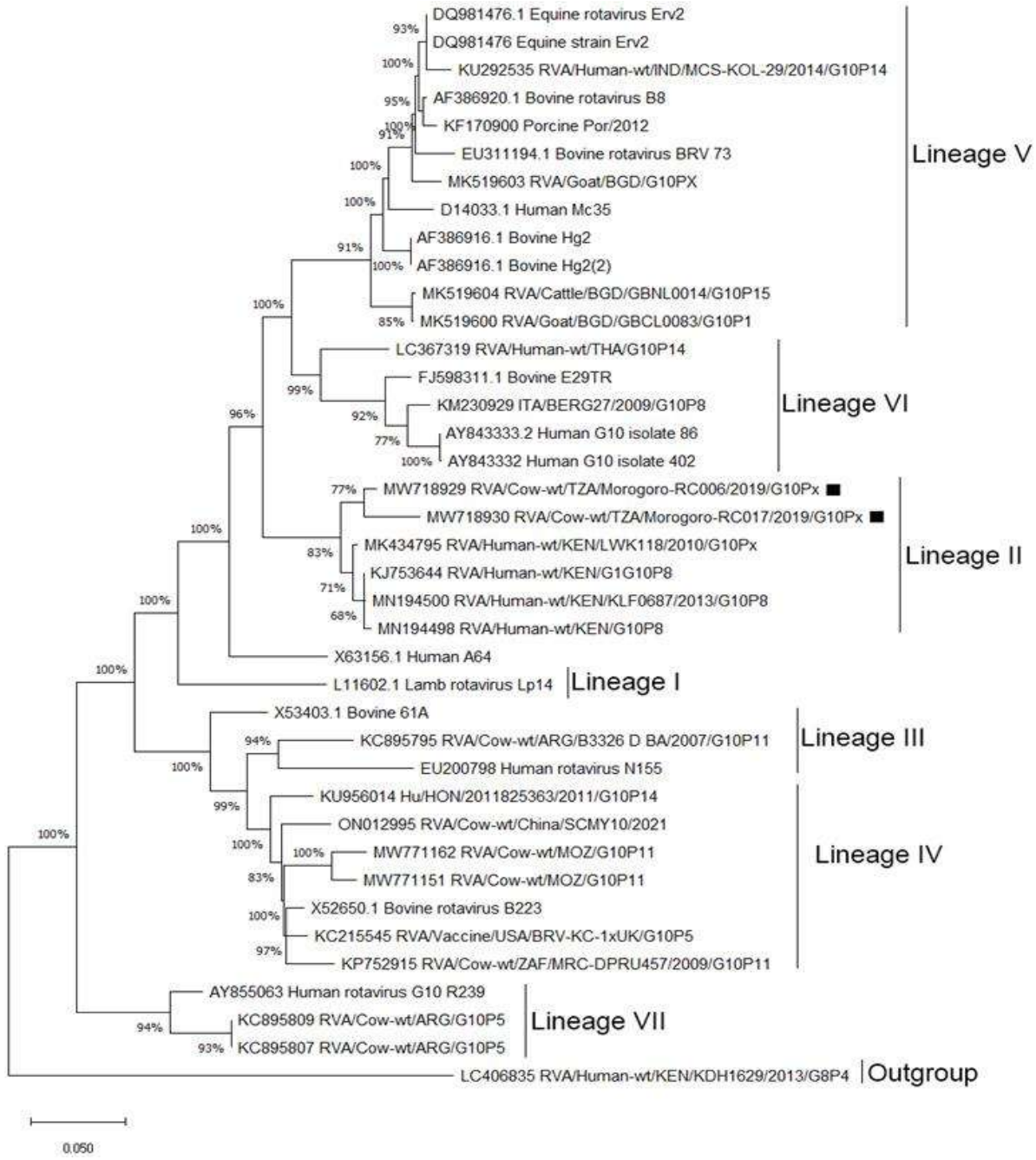

Figure 1. The nucleotide sequence phylogeny tree depicting the genetic relationship of VP7 gene of G10 genotypes, this study isolates are labelled with a black filled square.
Source: Authors

reassortment events and interspecies transmission. The occurrences of amino acid variation at the antigenic epitopes and N-linked glycosylation sites in comparison to the reference strain suggest potential of G10 genotype for escape neutralization antibodies. Because only a few genotypes were studied, and further research was recommended to gain a better understanding of rotavirus epidemiology, ecology, and evolution.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 2. The antigenic residues of VP7 (7-1a, 7-1b, and 7-2) present in genotype G10 strains of reference strain B223 and circulating strains were compared. Green highlights the amino acid residues in the circulating strains that differed from those in the reference strain.

	LN	7-1a																														
		87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
Bovine vaccine strain B223	2	T	T	N	N	E	W	T	S	Q	D	A	V	D	K	Q	N	T	R	D	A	R	N	S	S	L	S	E	A	G
RVA/Cow-wt/TZA/Morogoro-RC006/2019/G10P[x]	9	T	T	N	N	E	W	T	S	Q	D	T*	V	N*	I*	Q	N	T	G*	D	A	R	N	S	S	L	S	E	A	G
RVA/Cow-wt/TZA/Morogoro-RC017/2019/G10P[x]	9	T	T	N	S*	E	W	T	S	L*	D	T*	V	D	I*	H*	N	T	G*	D	A	R	N	S	S	L	S	E	A	G

Source: Authors

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African Journal of Microbiology Research

Full Length Research Paper

16S rRNA gene sequence analysis of the microbial community on microplastic samples from the North Atlantic and Great Pacific Garbage Patches

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The exponential increase in plastic production has led to their accumulation in the environment, particularly in oceans, polluting these environments from the shore to the open ocean and even sea ice in the pole regions. Microbial communities were compared on plastic particles, known as "Plastisphere", collected from the Atlantic and Pacific ocean gyres in the Summer of 2019 and subsequently inspected for potential plastic degraders. A 16S rRNA amplicon sequencing approach was applied to decipher differences and similarities in colonization behaviour between these two gyres. Polyethylene (PE) and polypropylene (PP) plastic samples were retrieved and investigated. We found that microbes differed significantly between the two oceans and identified thirty-two differentially abundant taxa at the class level. Proteobacteria, Cyanobacteria and Bacteroidota were the most prominent relative abundant phyla in the two oceans. Finally, according to the current literature, we found 40 genera documented as potential plastic degraders. This study highlights the importance of the biogeographical location with respect to microbial colonization patterns of marine plastic debris, differing even in the open oceans. Furthermore, the wide distribution of potential plastic-degrading bacteria was shown.

Key words: Plastisphere, microbial communities, plastic degraders.

INTRODUCTION

4,8 to 12,7 million metric tons of plastics are estimated to enter the ocean yearly, mostly from land (Jambeck et al., 2015). Dris et al. (2016) related an atmospheric fallout between 2 and 355 particles/m^2/day. Microscopic plastic fragments and fibres (microplastics) are widespread in the oceans. They have accumulated in the pelagic zone and sediments resulting from the degradation of macroplastic items (Thompson et al., 2004). The ubiquitous plastics in the ocean could harm the marine environment and humans through the food web. Evidence showed that microplastics could act as passive samplers for toxic compounds such as persistent organic pollutants (e.g., industrial chemicals, dioxins, pesticides) and heavy metals from seawater, leading to an increased

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negative impact on the biota (Mato et al., 2001; Horn et al., 2019). Besides that, the potential accumulation of microplastics in the food chain, especially in fish and shellfish, also exposes human consumers to these adsorbed chemicals (Kershaw and Rochman, 2014). Lusher et al. (2013) reported that 36% of pelagic and demersal fish collected from the English Channel had microplastics in their gastrointestinal tract.

Outreaches, national and international laws, policies, and conventions have been discouraging the use of plastic and its release into the environment to face plastic pollution. The African continent is at the forefront of legislative actions against plastic pollution. For instance, Rwanda has banned non-biodegradable plastic since 2008 and single-use plastics in 2019. The ban prohibited the manufacturing, use, import and sale of plastic carrier bags and forbade travellers into Rwanda to come with such products. Nigeria announced a ban on plastic bags in 2013, and in 2020, it strengthened its legislation by including a fine of 1072,16 Euro or three years jail term for any store found giving plastic bags to customers. In Botswana, a minimum thickness for bags was established and mandated that retailers apply a minimum levy to thicker bags, which would support government environmental projects. Kenya has the strictest ban on single-use plastic globally and in protected areas (Greenpeace, 2021).

Plastic is a high molecular weight synthetic polymer of a long chain of hydrocarbons derived from petrochemicals (Ahmed et al., 2018). With swift development in molecular techniques, research focused on microbial communities living on plastics and their ability to degrade hydrocarbons. The biological deterioration of plastic pollutants depends on many factors: surface area, functional groups, molecular weight, hydrophobicity, melting temperature, chemical structure, crystallinity, etc. (Okada, 2002). Microbial degradation of plastic involves many steps: biodeterioration, bio-fragmentation, assimilation, and mineralization (Purohit et al., 2020**)**.

Zettler et al. (2013) coined the term "Plastisphere" to describe biofilm-forming communities on marine plastic debris. They collected marine plastic debris at multiple locations in the North Atlantic to analyze the microbial consortia attached to it. They found diverse microbial communities, including heterotrophs, autotrophs, predators, and symbionts, which they called a 'Plastisphere'. Coons et al. (2021) investigated plastic-type and incubation locations in the Atlantic and Pacific oceans, focusing on shore locations as drivers of marine bacterial community structure development on plastic via 16S rRNA gene amplicon analysis. They found that incubation location was the primary driver of the coastal Plastisphere composition. The bacterial communities were consistently dominated by the classes Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia, irrespective of sampling location or substrate type.

Similarly, in 2015, Zettler et al. (2013) used next-generation DNA (Deoxyribonucleic Acid) sequencing to characterize bacterial communities from the Pacific and Atlantic oceans. Their objective was to determine whether the composition of Plastisphere communities reflects their biogeographic origins. They found that these communities differed between ocean basins and, to a lesser extent, between polymer types and displayed latitudinal gradients in species richness.

For this work, plastic particles were collected from the North Atlantic and the Great Pacific Garbage Patches in 2019 to compare microbial communities from the Atlantic to the Pacific, as well as looking for potential plastic degraders. The North Atlantic and the Great Pacific Garbage Patches are the biggest current patches, with a density of 10^6 km^{-2} (Eriksen et al., 2014) and 96 400 million metric tons of plastic (Ritchie and Roser, 2018), respectively. To reach our goal, we utilized 16S rRNA gene amplicon sequence analysis to study the microbial community of these microplastic samples.

The authors were able to point out differences, as well as taking into account the plastic polymer type, of which especially PP and PE are understudied to date.

MATERIALS AND METHODS

Plastics collection

The samples were collected in the North Atlantic and Great Pacific Garbage Patches. The pieces from the Atlantic were collected between 26-08-2019 and 04-09-2019 during the POS536 cruise project 'Distribution of Plastics in the North Atlantic Garbage Patch' (DIPLANOAGAP) aboard the German research vessel (R/V) Poseidon. A Neuston catamaran onboard R/V Poseidon, equipped with a microplastic trawl net (mesh size 300 µm, mouth opening 70 cm x 40 cm) was used to collect the plastic samples from the sea surface. After each tow, all microplastic fragments were removed from the trawl sample and conserved in a saturated ammonium sulphate solution (700 g/l ammonium sulfate, 20 mM sodium citrate, 25mM EDTA, pH 5,2). This solution precipitated all proteins, preventing DNA and RNA degradation for an extended time, even at room temperature. Verification of plastic type by Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) spectroscopy analysis was subsequently performed by TUTECH GmbH in Hamburg, Germany.

Another cruise project, MICRO-FATE, aboard another German R/V, the Sonne (SO268/3), between 05-06-2019 and 27-06-2019, was used to collect plastic samples at the sea surface in the Great Pacific garbage patch. Plastics were collected using a scoop net sampling method. The plastic surfaces were scraped using a flame-sterilized scalpel, and biofilms were transferred into microcentrifuge tubes. The sampling area was 16 × 16 mm, and tubes were immediately frozen in liquid nitrogen. At each station, 1 litre of pacific water was filtered through a 3 µm filter (3 µm Isopore TSTP 04700 Millipore, Merck KGaA, Frankfurt, Germany) and a 0,22 µm filter (0.22 µm Isopore GTTP04700 membrane filters Millipore, Merck KGaA, Frankfurt, Germany). Also, the filters were transferred to microcentrifuge tubes and immediately frozen in liquid nitrogen.

Extraction of nucleic acids from the samples

For Atlantic samples, sections of the different plastic samples were cut with a sterile scalpel and placed into 2 ml MP Biomedicals™

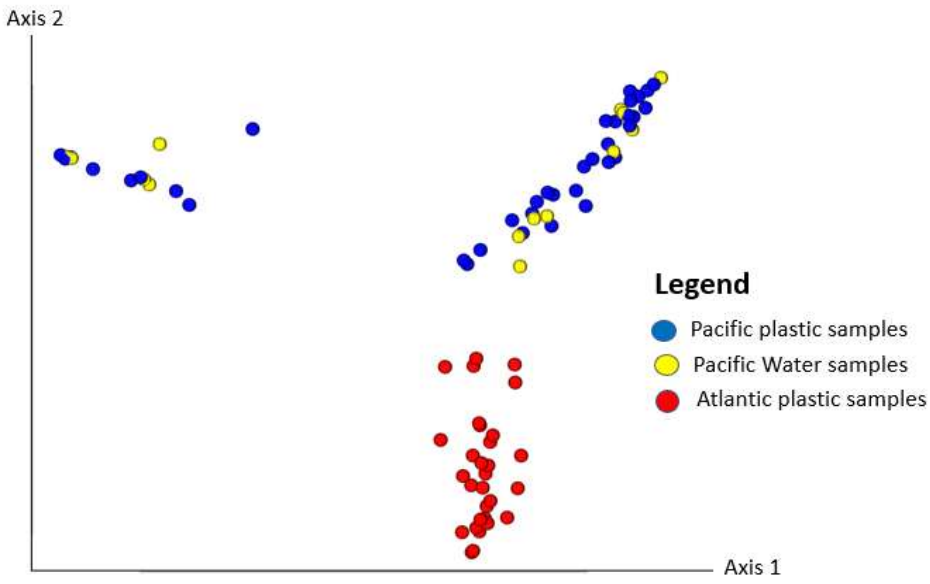

Figure 1. Principal coordinates analysis (PCoA) of all samples showing a specific clustering pattern of the associated microbial communities from the different oceans and samples. The phylogenetic distances calculated within the dataset indicate three clusters showing their level of relatedness.
Source: Authors.

Lysing Matrix E tubes (MP Biomedicals, Eschwege, Germany). Then physically disrupted using a bead-beating technique, with a single cycle of 30s at a speed of 5500 rpm in a FastPrep homogenizer (Qiagen, Hilden, Germany). The DNA extraction from the lysis product was then performed using the Qiagen AllPrep DNA/RNA Minikit according to the manufacturer's instructions. The quality and quantity of the DNA extraction were assessed using a NanoDrop Spectrophotometer (Desjardins and Conklin, 2010). The 16S rRNA gene was amplified with the primer pair 27F and 1492R. The sequencing of the V3-V4 region of the 16S rRNA gene was performed with v3 chemistry on a MiSeq Illumina sequencing platform at the Competence Centre for Genomic Analysis (CCGA) Kiel, Germany after the PCR products were visually assessed using 1% gel electrophoresis. For amplicon sequencing, the amplification of the V3-V4 hypervariable region of the 16S rRNA gene was accomplished using primer pair 341F (50-CCTACGGGAGGCAGCAG-30; Muyzer et al., 1993) and 806R (50-GGACTACHVGGGTWTCTAAT-30; Caporaso et al., 2011). Raw reads were archived in NCBI under the BioProject number PRJNA901861.

For Pacific samples, DNA was extracted from the biofilm pellets and water filters using the Macherey Nagel DNA Nucleo spin soil kit (Nucleo Spin TM Soil kit Macherey-Nagel TM, Düren, Germany) according to the manufacturer's instructions. DNA concentration was measured using a nano Qubit (ThermoFisher). Next-generation Illumina Sequencing was performed on an Illumina MiSeq platform using a V3 (300bp paired-end read) kit with a sequencing amount of 20 million reads, using the 341F (CCTACGGGNGGCWGCAG) and 785R primer set (GACTACHVGGGTATCTAAKCC). Raw reads were archived in NCBI under the BioProject number PRJNA837054.

Quantitative insights into microbial ecology (QIIME2) pipeline

The Raw amplicon sequences were then processed using the open-source Quantitative Insights into Microbial Ecology (QIIME2, version 2020.11) following a pipeline developed by Busch et al. (2021).

In brief, the *cutadapt* plugin was used to trim forward primers, heterogeneity spacers from forward-only single-end fastq files (Martin, 2011) and the *qualityfilter* plugin (Bokulich et al., 2013) was used to check the quality of the demultiplexed reads. An interactive plot served to visualize these results and to determine an appropriate truncation length. Then, the reads were truncated through the DADA2 algorithm to produce a total read length of 270 nucleotides. That truncation significantly increased the quality of the reads, reduced the overlap between forward and reverse reads, and allowed us to use only forwards reads for the analysis. Before the truncation, the reads were denoised using the *denoise-single* method of the DADA2 algorithm (Callahan et al., 2016), which removed chimeric sequences and inferred sample composition using a parametric error model.

The amplicon sequence variants (ASV; Callahan et al., 2017) were classified at 80% confidence level using the most recent SILVA 138 16S rRNA gene reference database (Quast et al., 2013; Yilmaz et al., 2014). Common eukaryotic contaminants (chloroplasts, mitochondria) and unassigned sequences were removed using the *filter-features* method of the *featuretable* plugin, and the resulting dataset was rarefied to 8000 sequences. Alpha rarefaction curves have an excellent saturation for 8000 sequences. A phylogenetic backbone tree was built using FastTree (Price et al., 2009, 2010) and MAFFT (Katoh and Standley, 2013) alignment through the *phylogeny* plugin. The resulting tree was used to compute core diversity metrics which served to compute downstream analyses along with an alpha-rarefaction curve *via* the *diversity* plugin.

A PCoA plot was produced within QIIME2 and for the ASV distribution between the different sampling domains the 'Venn diagram' package in R was utilized (Figures 1 and 2). Sunburst charts displaying the taxonomic distribution of reads were designed with the 'plotly.express' package in Python (Figures 3 and 4).

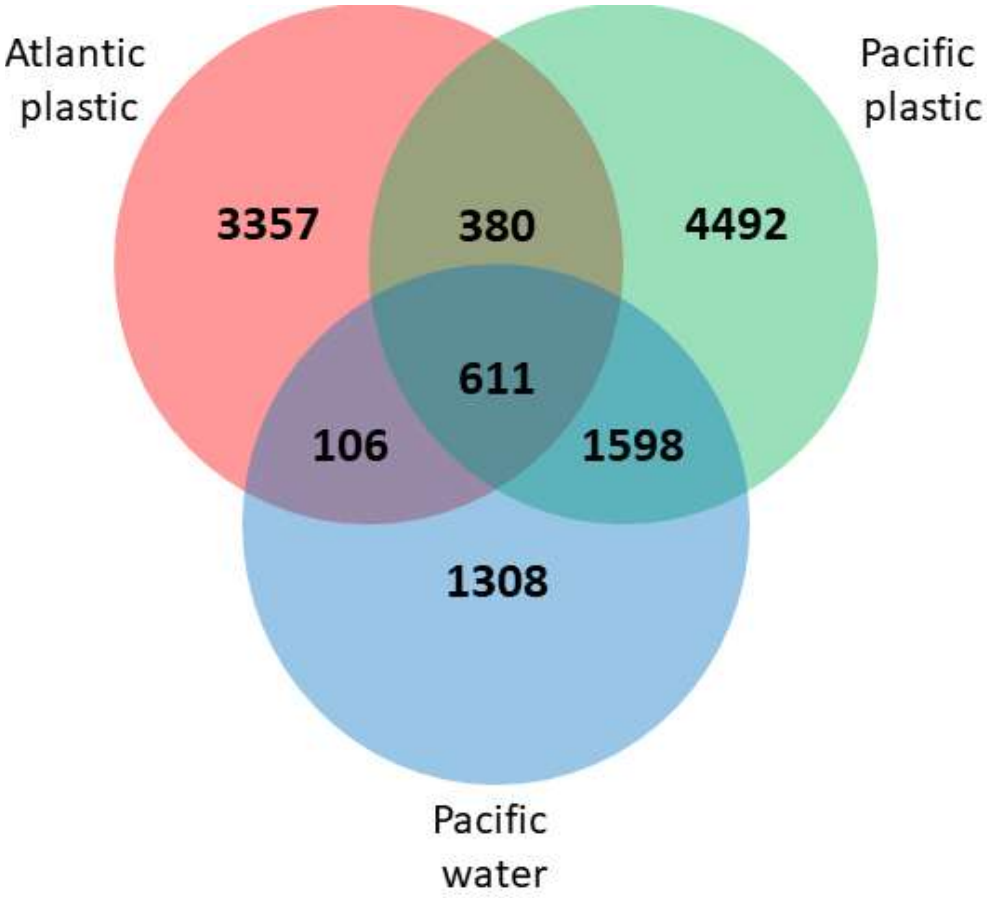

Figure 2. Distribution of ASVs between the different sample types. Unique and shared ASVs between Atlantic plastic, Pacific plastic, and Pacific water samples.
Source: Authors.

Alpha and Beta diversity measures

The alpha diversity was investigated according to unique ASVs per sample (species richness), taking into consideration the number of times each ASV occurs in the sample (Pielou's evenness) and the phylogenetic relatedness of each sample community (Faith's PD). *'Qiime diversity alpha-group-significance*' plugin in QIIME2 was used to assess the diversity within each area. The results were displayed through Kruskal-Wallis (all groups) and Kruskal-Wallis (pairwise) results.

Non-phylogenetic (evenness) and phylogenetic (Faith's PD) diversity indices were visualized using the online tool QIIME2 view (https://view.qiime2.org/). Eventually, if the comparison revealed a significant difference in microbial diversity, Kruskal-Wallis pairwise was considered among groups to see where the difference lies.

Beta diversity measures assessed the differences between groups following the different parameters. 'Qiime diversity beta-group-significance' plugin in QIIME2 was used for this analysis. The analysis was performed using the non-metric multidimensional scaling method (NMDS; Kruskal, 1964) with a sample-wise unweighted UniFraq distance matrix (Lozupone and Knight, 2005). Each group was assessed based on its distance from the other groups in QIIME2; boxplots were displayed simultaneously with the PERMANOVA results and pairwise PERMANOVA results between groups. The PERMANOVA group significance and pairwise tests were run simultaneously through the *betagroup-significance* method (non-parametric MANOVA; Anderson, 2001) of the diversity plugin with an unweighted UniFraq matrix and 999 permutations as input.

We adopted the standard significant measure, p-value = 0.05, for these statistical analyses. All the p-values below this standard describe a significant difference between the compared parameters and vice versa.

Different taxonomic level analysis

The feature ASVs table was exported in biom format in QIIME2. Subsequently, the taxonomy metadata file was added to the biom file and exported in TSV file format using '*biom convert*' plugin in QIIME2. Further analyses outside the QIIME2 environment, such as the share of ASVs between the samples, were performed using the resulting TSV file table. Besides that, the same feature table was collapsed at the genus level (to perform the sunburst plots, which helped to display microbial communities on plastics) and the class level (to plot the differentially abundant taxa) using the '*qiime taxa collapse*' plugin. The Linear discriminant analysis (LDA) effect size (LEfSe) helped to plot the differentially abundant classes between the Atlantic and the Pacific Plastisphere, utilizing 'galaxy online' (https://huttenhower.sph.harvard.edu/galaxy/). The level 3 data was used, arranged within Excel (according to the different oceans) and imported into Galaxy for LEfSe analysis. The analyses were performed on the microbial community relative abundance data in both oceans. Grouped data were first analyzed using the Kruskal-

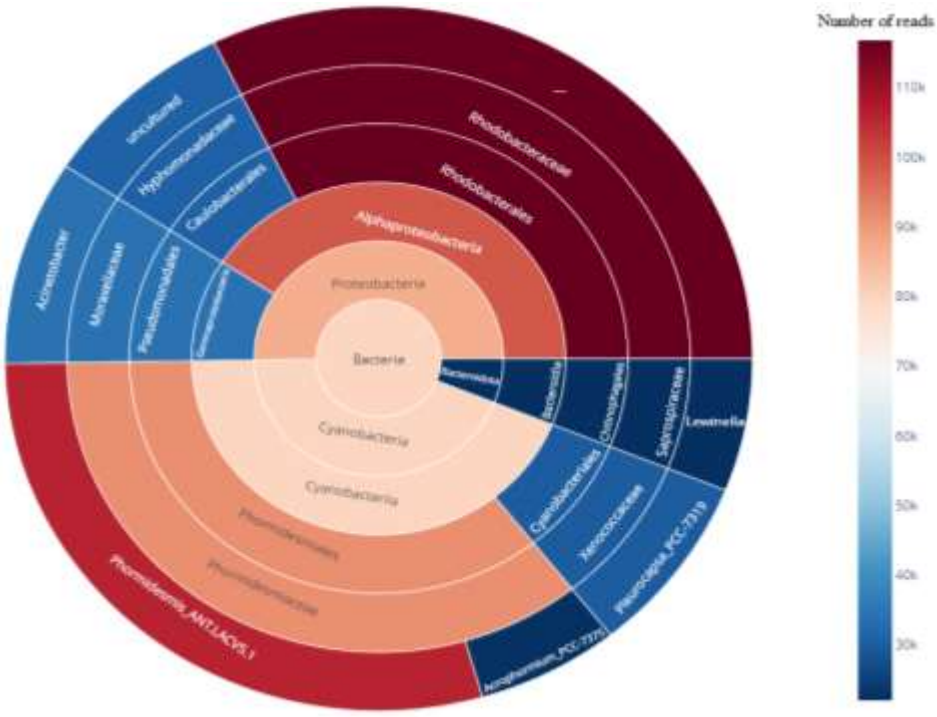

Figure 3. Reads and taxonomic affiliation of recurring communities on the Atlantic Plastisphere. Sunburst chart displaying the affiliations of genera that reached values above 20000 reads. Each plot crown represents one taxonomic level from the Kingdom to the genus.
Source: Authors.

1. Actibacterium
2. Limibaculum
3. Uncultured
4. Silicimonas
5. Hyphomonadaceae
6. Caulobacterales
7. Metagenome
8. AEGEAN-169_marine_group
9. Rhodospirillales
10. Sphingomonadaceae
11. Sphingomonadales
12. SAR116_clade
13. Puniceispirillales
14. Rhizobeaceae
15. Rhizobiales
16. SAR86_clade
17. Gammaproteobacteria
18. Pleurocapsa_PCC-7319
19. Xenococcaceae
20. Phormidesmis_ANT.LACV5.1
21. Acrophormium_PCC-7375
22. Phormidesmiaceae
23. Phormidesmiales
24. Actinobacteria
25. Acidimicrobiia
26. Actinomarinales
27. Actinomarinaceaea
28. Candidatus_Actinomarina
29. Bacteroidota
30. Bacteroidia
31. Flavobacteraceae
32. Flavobacteriales
33. Muricauda

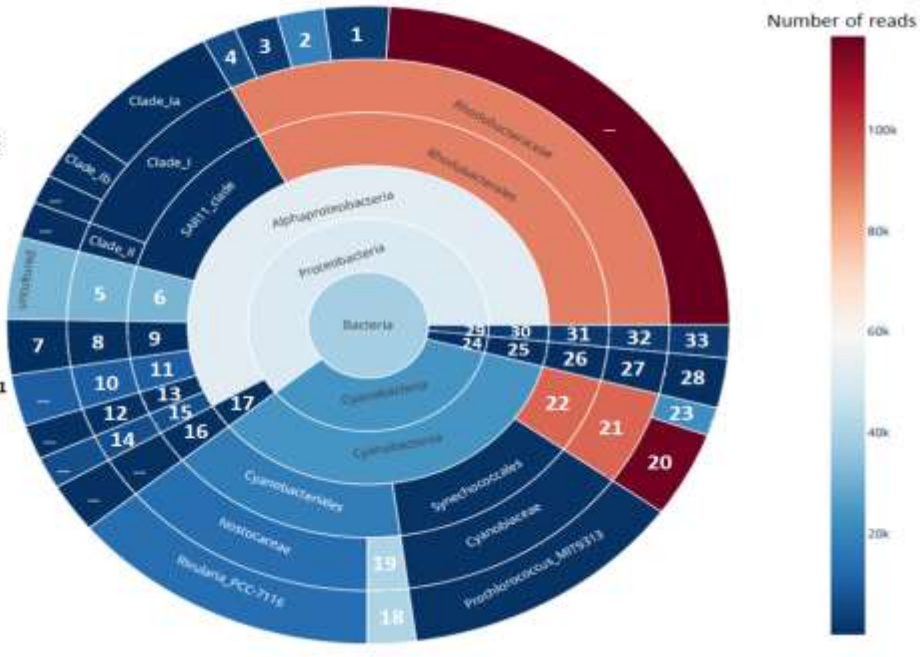

Figure 4. Reads and taxonomic affiliation of recurring communities on the Pacific Plastisphere. Sunburst chart displaying the affiliations of genera that reached values above 20000 reads. Each plot crown represents one taxonomic level from the Kingdom to the genus.
Source: Authors.

Table 1. Taxonomic rank abundance distribution per sample type and percental display of unclassified ASVs per taxonomic rank.

Taxonomic level	Atlantic plastic		Pacific plastic		Water	
	Classified	%Unclassified	Classified	%Unclassified	Classified	%Unclassified
Kingdom	3	0	3	0	3	0
Phylum	35	0.51	33	0.12	27	0.13
Class	74	0.57	74	0.26	55	0.21
Order	161	4.19	172	2.30	142	2.91
Family	241	5.75	252	5.35	206	5.67
Genus	369	34.09	400	35.33	323	38.56

Source: Authors

Wallis test with a significance threshold of 0,05 to determine if the data was differentially distributed between groups.

RESULTS AND DISCUSSION

Samples collected comprised 68 microplastic pieces from the North Atlantic and the Great Pacific Garbage Patches as well as 14 water samples from the Great Pacific Garbage Patch. The North Atlantic Garbage Patch accounted for 30 plastic samples composed of 25 PE (polyethylene) and 5 PP (Polypropylene) particles, according to FTIR analysis. In contrast, the Great Pacific Garbage Patch accounted for 38 plastic samples, composed of 28 PE and 10 PP (Supplementary Table S1).

Processing all samples in QIIME2 yielded 11852 demultiplexed unique ASVs. Pacific plastic accounted for 7081 ASVs displaying higher diversity than Atlantic plastic which yielded 4454 ASVs and, Pacific water displaying the lowest number of ASVs (3623 ASVs). The Pacific plastisphere displayed the highest number of different taxa at almost all taxonomic levels except the phylum level (Table 1). Here, the Atlantic plastisphere displayed the highest number of phyla with 35, whereas Pacific plastic contained 33 phyla and Pacific water only 27 phyla. Overall, the Pacific water displayed the lowest number of different taxa irrespective of the taxonomic level, which might hint towards a microhabitat formation on the plastic particles as they travel across the oceans and enrich their community along the way. These results also show an increasing proportion of unclassified taxa as one moves from the phylum level to the genus level, which underlies the underexploration of marine bacterial diversity. The Shannon diversity indices values are between 4,88 and 8,75 in the individual samples (Supplementary Table S1), with no apparent large differences between the different sample types and locations.

The principal coordinates analysis (PCoA) of all samples

A PCoA plot, grouping all the samples, resulted in three distinct clusters (Figure 1). It shows that the communities of each area are distinct from the other locations. However, in the Pacific Ocean, two clusters were formed, which could explain that an occurrent factor influences the diversity of the microbes. Interestingly, the two clusters contain each samples from the two different sampling domains Pacific plastic and Pacific water. The outgroup in the Pacific Ocean are the only samples with a noticeable amount of Archaea belonging to the class of Thermoplasmata (0.6% of the reads). These Archaea were investigated by Gupta et al. (2021), and were shown as acidophiles.

Comparison of the ASVs distributions between the different sampling domains and oceans

The three different sampling domains, being Atlantic plastic, Pacific plastic and Pacific seawater, represented by a total 11852 unique ASVs, shared only 611 ASVs (5%). 380 ASVs (3%), were exclusively shared between Atlantic plastic and Pacific plastic. 106 ASVs (1%) were shared between the Atlantic plastisphere and the Pacific water.1598 ASVs (13%) were found in the Pacific Plastisphere and the Pacific water. Conversely, 4492 (38%) of the ASVs were unique to the Pacific plastics, 3357 ASVs (29%) to the Atlantic plastics and 1308 ASVs (11%) to the Pacific water (Figure 2). A negligible proportion of ASVs is shared between the two oceans with the investigated three different sampling domains (8%), while each ocean showed a big proportion of unique ASVs, suggesting a profound difference between their communities (92%).

Microbial composition on the Atlantic plastics

From the analysis, the highest relative abundances were bacteria (99.91%). Three bacterial phyla accounted for more than 90% of the relative abundance. Verrucomicrobiota, Bdellovibrionota and Firmicutes accounted for more than 1% each, while 29 other phyla (including bacterial, archaeal and eukaryal phyla)

accounted for 4.70% of the community (each of these 29 phyla accounted for below 1% of the relative abundance).

Among the abundant minor domains, Eukaryota (0.09%) were represented by the phyla Amorphea (0.08%) and SAR (0.002%) and the classes of Obazoa and Alveolata. Likewise, the reads of Archaea (0.0002%) were represented by the phylum of Nanoarchaeota and the class of Nanoarchaeia**.**

Proteobacteria, Cyanobacteria and Bacteroidota were the three most abundant groups at the phylum level (Figure 3). The occurrent communities include Alphaproteobacteria (34.60%), reported as early colonizers; Bacteroidia (17.04%), reported as secondary colonizers and Gammaproteobacteria (10.9%), later-stage colonizers at the class level, according to a recent 16S rRNA gene amplicon data meta-analysis from 35 Plastisphere studies, which revealed the successive colonization of the Plastisphere (Wright et al., 2020). So, Gammaproteobacteria's presence suggests the maturity of the biofilm, indicating that the plastics have been drifting for quite some time. Meanwhile, members of the phylum Cyanobacteria have been reported as abundant components of plastic debris communities (Salta et al., 2013) highly represented on PP and PE items (Zettler et al., 2013).

Other communities at the Family level include bacteria that prefer a surface-attached lifestyle, such as Saprospiraceae (McIlroy and Nielsen, 2014), Hyphomonodaceae, known to be biofilm formers (Abraham and Rohde, 2014) and Rhodobacteriaceae as opportunistic colonizers (Dang and Lovell, 2016). At the genus level, *Lewinella* and *Acinetobacter* were described as potential plastic degraders (Table 3).

Microbial community composition on the Pacific plastics

After processing, 99,38% of the reads belonged to the domain of Bacteria. Three phyla were most abundant, with almost 91% of the total read count. The other important relative abundant phyla were classified as Planctomycetota, Actinobacteriota and Verrucomicrobiota. They accounted for 6.23% of the total reads. Twenty-seven phyla stemming from Bacteria, Archaea and Eukaryota accounted for 2,76% (each of the 27 recorded below 1% of the reads).

Among the small percentage reads, Archaea (0.62%) showed more diversity in the Pacific than within the Atlantic and were represented by the phyla Thermoplasmatota (0.62%), Nanoarchaeota (0.00058%) and Halobacterota (0.00008%).

At the class level, Archaea were represented by Thermoplasmata, Nanoarchaeia and Methanosarcinia. Meanwhile, Eukaryota (0.00018%) displayed less diversity than within the Atlantic. They were represented by one phylum, SAR and one class, Stramenopiles**.**

Proteobacteria, Cyanobacteria, Bacteroidota and Actinobacteria were the most abundant groups at the phylum level (Figure 4). In addition to the three highest abundant phyla reads, the Pacific Plastisphere recorded Actinobacteria (2.31%), which have been reported as an abundant component of plastic debris communities (Salta et al., 2013; Pinto et al., 2019). Herein, Cyanobacteria and Proteobacteria showed more diversity than in the Atlantic. Gammaproteobacteria were also present, suggesting the maturity of the Pacific biofilms. As such, the Pacific plastics have been drifting for quite some time. At the family level, the figure shows the presence of Hyphomonadaceae and Rhodobacteraceae but not Saprospiracea as in the Atlantic Plastisphere. Instead, Flavobacteraceae, bacteria that prefer surface-attached lifestyles, were present herein.

Microbial community composition in the Pacific water

Pacific water sample analysis was performed to compare microbial communities on Pacific plastic and its surrounding water. Many studies showed that plastic communities differ from surrounding water communities.

From the analysis, bacteria were the most prominent domain, with 99.62%. Its phyla Proteobacteria, Cyanobacteria and Bacteroidota accounted for more than 91% of the relative abundances. Actinobacteria, Verrucomicrobiota, Planctomycetota and Patescibacteria accounted for 7.11%. The rest (20 phyla), stemming from Bacteria, Archaea and Eukaryota, accounted for 1.83% of the reads.

Archaea in water (0.37%) were represented by the phylum of Thermoplasmatota and the class of Thermoplasmata. Meanwhile, Eukaryota (0.0019%) were represented by the phylum of Amorphea and the class of Obazoa.

Among the occurring phyla between Pacific Plastisphere and Pacific water, Dependentiae (0.005%), PAUC34f (0.002%), Nanoarchaeota (0.0004%, from Archaea), SAR (0.0001%, from Eukaryota), Latescibacterota (0.0001%), Fibrobacterota (0.0001%) and Halobacterota (0.00007%) were found only on Pacific plastic. Amorphea (0.0005%, from Eukaryota) was found only in water. That could probably hint toward the specificity of certain microorganisms for specific substrates.

Statistical analysis of the microbial community diversity composition of the samples

The statistical analysis of the samples showed a non-significant difference in microbial community diversity within the Atlantic area based on plastic polymer types as well as within the Pacific area. The p-values are greater than 0.05, as shown in Table 2. Indeed, some studies

Table 2. Statistical analysis of the samples: table displaying p-values from Kruskal-Wallis and PERMANOVA analysis.

		Atlantic plastisphere	Pacific plastisphere	Pacific plastics and water	Atlantic and pacific plastics
Alpha diversity	Non-phylogenetic measure				
	Considering the polymer types	0.8	0.6	0.96	0.82
	Regardless of the polymer types			0.88	0.38
	Phylogenetic measure				
	Considering the polymer types	0.67	0.57	0.4	0.002
	Regardless of the polymer types			0.23	0.000063
Beta diversity	Considering the polymer types	0.35	0.84	0.8	0.001
	Regardless of the polymer types			0.59	0.001

Source: Authors

showed that the plastic polymer types have no effect in determining the Plastisphere community composition in mature biofilms (Oberbeckmann and Labrenz, 2020). So, these results confirm the maturity of the biofilms in the Atlantic and Pacific Plastisphere. Also, the p-values displayed (Table 2) while assessing the diversity between the Pacific plastics and its surrounding water showed no significance. The Pacific Plastisphere was not significantly more or less diverse than the microbial community in the Pacific water. Indeed, Oberbeckmann et al. (2014) suggested that communities at early times in the colonization process are more likely to reveal polymer-specificity, while communities that establish on different polymers should gradually converge over time as the biofilms mature (Harrison et al., 2011).

Meanwhile, the diversity assessment of the Atlantic and Pacific Plastisphere showed significant p-values for phylogenetic measures and beta diversity (Table 2). So, the communities in the Atlantic Plastisphere are significantly distinct from those in the Pacific Plastisphere. It confirms the results obtained by Amaral-Zettler et al. (2015) seven years ago on the same topic when assessing the diversity between Atlantic and Pacific communities. They found the same significance level (p-value = 0.001); distinct grouping based on the oceanic biogeographic zone (Atlantic versus Pacific).

Biogeography is incontestably a driver of microbial diversity. Similar results were also obtained by Coons et al. (2021) who found that biogeography influences Plastisphere community structure more than substrate type. Differences in the biofilm community composition are related to different factors.

Some previous studies have targeted temperature as the best predictor of bacterial diversity in surface waters (Ibarbalz et al., 2019). Regarding this study, the plastic particles were collected at the surface of different waters. They could have attracted microbial communities able to evolve at the various water surfaces.

Other studies showed that the substratum physicochemical properties (hydrophobicity, roughness, vulnerability to weather) and the surface chemodynamics (surface conditioning or nutrient enrichment) play a role in microbial diversity (Dang and Lovell, 2016). Besides physicochemical surface properties, it has been shown that the composition of biofilm communities associated with synthetic polymers differed significantly for different ocean basins (Amaral-Zettler et al., 2015) and underlay both seasonal and spatial effects, e.g., in North Sea waters (Oberbeckmann et al., 2014). The waters from the Atlantic and Pacific Oceans seem to have different physicochemical properties, which could have impacted the properties of the plastic we collected, especially since they lasted in the water.

Future studies on the same topic should include environmental parameters to determine the likely drivers of this difference in microbial diversity composition between the Atlantic and Pacific, which were not collected for the Atlantic samples. So, the pH (as it varies between the Atlantic and the Pacific), the dissolved oxygen, the salinity or the surface temperature (as it also varies between both oceans) could be responsible for this difference in microbial diversity between the

Atlantic and Pacific oceans.

Differentially abundant classes between the Atlantic and Pacific plastisphere

The above mentioned statistics showed that there is effectively a significant difference between the Atlantic and the Pacific microbial community diversity. Linear discriminant analysis (LDA) effect size (LEfSe) was used to predict the class level abundance differences between the Atlantic and the Pacific and so highlight which classes drive the community differences. It revealed 32 differentially abundant classes (LDA log score > ±2) between the Atlantic and the Pacific, as displayed in Figure 5. The dominant classes that made the difference between the Atlantic and the Pacific (Figure 5) belong to the phyla Proteobacteria, Bacteroidota, Planctomycetota, Bdellovibrionota, Bacilli, Verrucomicrobiota and Thermoplasmatota (from Archaea). SAR and Amorphea (from Eukaryota) were also part of the differentially abundant microorganisms.

The Atlantic contains 23 classes that are more abundant than in the Pacific, among which Alveolata and Obazoa are from Eukaryota. In comparison, the Pacific offers nine more abundant classes, among which is the class Thermoplasmata from Archaea. Among these 32 classes, 12 had an LDA score > ±3, including eight from the Atlantic (in ascending order Desulfuromonadia, TK17, Verrucomicrobiae, Anaerolineae, Bacilli, Bdellovibrionia, Gammaproteobacteria) and four from the Pacific (in ascending order Parcubacteria, Thermoplasmata, Planctomycetes, and Alphaproteobacteria). Alphaproteobacteria, Gammaproteobacteria, Bacteroidia had an LDA score > ±4. Thermoplasmata, ABY1 and Desulfovibrionia were unique to the Pacific, while Obazoa, endosymbiont_of_Ridgeia_piscesae, Vicinamibacteria, Alveolata and TK17 were unique to the Atlantic.

Potential plastic degraders within the studied plastisphere

The plastic-degrading potential of the Plastisphere community is an ongoing topic (Zettler et al., 2013). Exploring the present Plastisphere, 40 genera previously described to include hydrocarbon-degrading bacteria, as shown in Table 3, were deciphered. These genera represented 4.07% of the relative abundances of the whole Plastisphere and were shared in 4 phyla, five classes, 21 orders and 32 families. Proteobacteria was the most represented, with 22 genera.

Actinobacteria came after that with eight genera, Bacteroidota with seven genera and Firmicutes with three genera. Twelve genera were exclusively detected in the Atlantic and three in the Pacific, while 25 were shared between the two oceans. This hints towards potential utilization of floating plastics as carbon sources, but this remains to be proven with other methodologies.

The samples were composed of PP and PE. The distribution of PE-degrading microorganisms seems limited, although PP appears to be non-biodegradable. However, it was reported that *Acinetobacter* sp. 351 partially degraded lower molecular weight PE oligomers (the genus was found herein: 1.11%) upon dispersion. In contrast, high molecular weight PE could not be impaired (Tsuchii, 1980). The biodegradability of PE could be improved by blending it with biodegradable additives, photoinitiators or copolymerization (Griffin, 2007; Hakkarainen and Albertsson, 2004). A blending of PE with additives generally enhances auto-oxidation, reduces the molecular weight of the polymer, and then makes it easier for microorganisms to degrade the low molecular weight materials.

Meanwhile, the possibility of degrading PP with microorganisms has been investigated (Cacciari et al., 1993). In that study, it was shown that aerobic and anaerobic species with different catabolic capabilities could act in close cooperation to degrade polypropylene films. Some *Pseudomonas* (present in this Plastisphere) species were pointed out in the process of polypropylene degradation. Besides that, many species of *Pseudomonas* were indicated to degrade Polyethylene (Zheng et al., 2005), Polyvinyl chloride (Danko et al., 2004), while *Rhodococcus* was shown to degrade Polyethylene (Sivan et al., 2006).

Microbial communities associated with plastic degradation composition and species richness are influenced by spatiotemporal phenomena like habitats/geographical location, ecosystem, and seasonal variation (Kirstein et al., 2019; Pinto et al., 2019). Further, the physicochemical nature of plastics like polyethylene, polypropylene, polystyrene, also regulates this degradation (Pinnell and Turner, 2019). The composition and specificity of microbial assemblage associated with polyethylene (PE) and polystyrene (PS) in the marine aquatic ecosystem (coastal Baltic Sea) are indicated by an abundance of Flavobacteriaceae (*Flavobacterium*), Rhodobacteraceae (*Rhodobactor*), Methylophilaceae (*Methylotenera*), Plactomycetaceae (Planctomyces, *Pirellula*), Hyphomonadaceae (*Hyphomonas*), Planctomycetaceae (*Blastopirellula*), Erythrobacteraceae (*Erythrobacter*), Sphingomonadaceae (*Sphingopyxis*), etc. (Oberbeckmann et al., 2018). Kirstein et al. (2019) found that the microbial community composition associated with various plastics is significantly varying, and it is also changing with the different phases of the plastic degradation process. In this study, the genera, *Flavobacterium* (0.05%), *Hyphomonas* (0.01%) and *Erythrobacter* (0.29%) were precisely found to be associated with PE (0.27%), but also PP (0.2%).

The study presented microbial distribution patterns on plastics from two different major oceans and highlighted the need for close monitoring of plastic debris and their

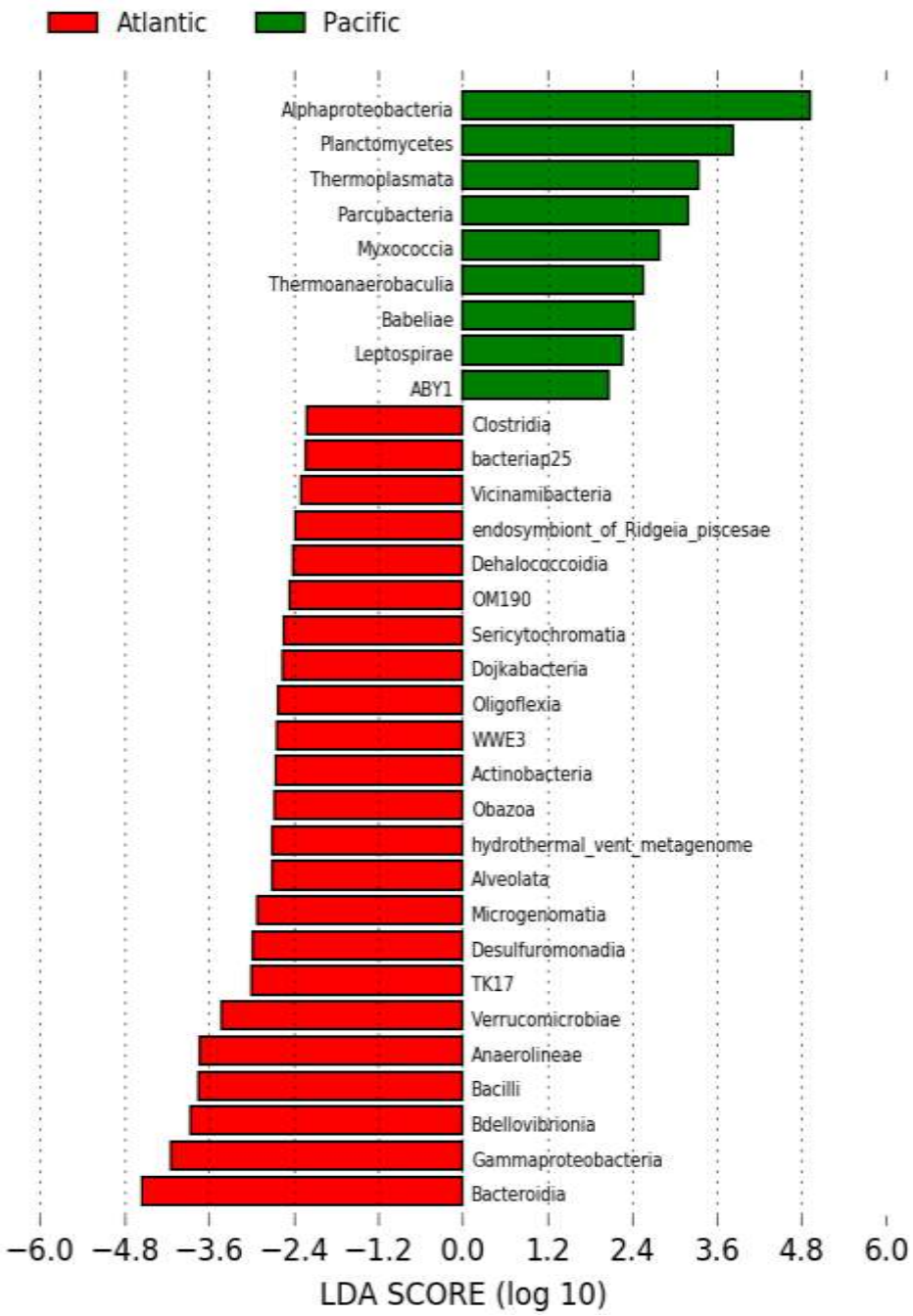

Figure 5. Differentially abundant classes between the Atlantic and the Pacific oceans. Linear discriminant analysis (LDA) effect size (LEfSe) results per ocean. Bar plots depict all classes which had an LDA log score > ±2 between all plastic samples (N = 68) in the Atlantic (n=30) or Pacific (n=38) oceans. The plot was made by utilizing galaxy online (https://huttenhower.sph.harvard.edu/galaxy/) with the tool LEfSe. Grouped data were first analyzed using the Kruskal-Wallis test with a significance threshold of 0.05 to determine if the data was differentially distributed between groups.
Source: Authors.

fate due to the individuality each plastic particle and its associated microbial community displays. Nonetheless, general patterns can be discerned, especially microbes commonly associated with hydrocarbon degradation can be found on most particles. These bacteria or better to say their 16S rRNA gene could be used, for example as

Table 3. Genera of potential plastic degraders within the studied Plastispheres. Genera in Bold are those detected only in one area, relative abundances are indicated in each ocean and on each plastic type, relative abundances below 0,01 are indicated as <0.01.

Genus and reference	Atlantic	Pacific	PP	PE	Total
Lewinella (Vaksmaa et al., 2021)	0.73	0.42	0.19	0.97	1.16
Acinetobacter (Chaineau et al., 1999)	1.10	0.01	0.41	0.69	1.11
Erythrobacter (Harwati et al. 2007)	0.08	0.29	0.18	0.18	0.37
Algimonas (Vaksmaa et al., 2021)	0.12	0.14	0.06	0.2	0.26
Vibrio (Hedlund and Staley, 2001)	0.18	0.032	0.03	0.18	0.21
Winogradskyella (Wang et al., 2014)	0.03	0.16	0.07	0.13	0.19
Tenacibaculum (Wang et al., 2014)	0.07	0.08	0.02	0.12	0.14
Alteromonas (Iwabuchi et al., 2002)	0.09	0.04	0.02	0.10	0.12
Brevundimonas (Chaineau et al., 1999)	0.1	0.002	0.05	0.05	0.1
Roseovarius (Peeb et al., 2022)	0.007	0.08	0.03	0.06	0.09
Pseudomonas (Le Petit et al., 1975)	0.06	<0.01	0.04	0.03	0.07
Hyphomonas (Yakimov et al., 2005)	0.04	0.01	<0.01	0.04	0.05
Flavobacterium (Stucki and Alexander, 1987)	0.05	<0.01	0.01	0.04	0.05
Fabibacter (Wang et al., 2014)	0.02	<0.01	<0.01	0.02	0.03
Dokdonia (González et al., 2011)	0.02	<0.01	<0.01	0.014	0.02
Stenotrophomonas (Juhasz et al., 2000)	0.02	-	0.01	<0.01	0.02
Marinobacter (Gauthier et al., 1992)	<0.01	0.01	<0.01	0.01	0.01
Halomonas (Wang et al., 2007)	<0.01	<0.01	<0.01	0.01	0.01
Oleiphilus (Golyshin et al., 2002)	0.01	-	-	0.01	0.01
Methylobacterium-Methylorubrum (Bodour et al., 2003)	<0.01	<0.01	<0.01	<0.01	0.01
Staphylococcus (Saadoun et al., 1999)	<0.01	<0.01	<0.01	-	<0.01
Hyphomicrobium (Ozaki et al., 2006)	-	<0.01	<0.01	<0.01	<0.01
Corynebacterium (Chaineau et al., 1999)	<0.01	<0.01	<0.01	<0.01	<0.01
Pseudoxanthomonas (Yue et al., 2021)	<0.01	-	<0.01	<0.01	<0.01
Chryseobacterium (Szoboszlay et al., 2008)	<0.01	<0.01	<0.01	<0.01	<0.01
Thalassospira (Kodama et al., 2008)	-	<0.01	<0.01	<0.01	<0.01
Alkanindiges (Bogan et al., 2003)	<0.01	-	<0.01	<0.01	<0.01
Alcanivorax (Yakimov et al., 1998)	<0.01	<0.01	-	<0.01	<0.01
Micrococcus (Ilori et al., 2000)	<0.01	<0.01	<0.01	<0.01	<0.01
Kocuria (Dashti et al., 2009)	<0.01	-	-	<0.01	<0.01
Rhodococcus (Meyer et al., 1999)	<0.01	-	<0.01	<0.01	<0.01
Methylophaga (Mishamandani et al., 2014)	-	<0.01	-	-	<0.01
Oleispira (Yakimov et al., 2003)	<0.01	<0.01	-	<0.01	<0.01
Mycobacterium (Willumsen et al., 2001)	<0.01	<0.01	<0.01	<0.01	<0.01
Nocardioides (Hamamura and Arp, 2000)	<0.01	-	<0.01	<0.01	<0.01
Arthrobacter (Le Petit et al., 1975)	<0.01	-	-	<0.01	<0.01
Actinomyces (ZoBell, 1946)	<0.01	-	<0.01	<0.01	<0.01
Achromobacter (Le Petit et al., 1975)	<0.01	-	-	<0.01	<0.01
Lactobacillus (Floodgate, 1984)	<0.01	-	-	<0.01	<0.01
Bacillus (Li et al., 2008)	<0.01	-	-	<0.01	<0.01

Source: Authors

biomarkers in sensor systems to detect high micro- and nanoplastic pollution that is not readily visible like the particles collected for this study. Another aspect of huge societal concern is the transport of pathogenic bacteria on plastic particles, for which no evidence was found in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Samples used in this study with their respective geographic location, plastic type and shannon diversity indice indicated.

Sample	Ocean	Plastic type	Shannon diversity
P_1	Pacific Ocean	LDPE	6.15
P_2	Pacific Ocean	HDPE	6.93
P_3	Pacific Ocean	PE	7.48
P_4	Pacific Ocean	PE	5.97
P_5	Pacific Ocean	PE	6.88
P_6	Pacific Ocean	PE	5.36
P_7	Pacific Ocean	PE	5.61
P_8	Pacific Ocean	PE	6.01
P_9	Pacific Ocean	PE	6.62
P_10	Pacific Ocean	PP	6.97
P_11	Pacific Ocean	PE	6.96
P_12	Pacific Ocean	PE	6.69
P_13	Pacific Ocean	PP	5.33
P_14	Pacific Ocean	PE	6.96
P_15	Pacific Ocean	PE	7.26
P_16	Pacific Ocean	PE	7.15
P_17	Pacific Ocean	PE	6.48
P_18	Pacific Ocean	PP	6.72
P_19	Pacific Ocean	PP	5.83
P_20	Pacific Ocean	PP	5.96
P_21	Pacific Ocean	HDPE	5.58
P_22	Pacific Ocean	LDPE	7.41
P_23	Pacific Ocean	PE	6.41
P_24	Pacific Ocean	PE	6.85
P_25	Pacific Ocean	PE	7.03
P_26	Pacific Ocean	PP	6.92
P_27	Pacific Ocean	PE	7.51
P_28	Pacific Ocean	PE	5.06
P_29	Pacific Ocean	PP	6.46
P_30	Pacific Ocean	PP	7.24
P_31	Pacific Ocean	PE	6.64
P_32	Pacific Ocean	PE	6.57
P_33	Pacific Ocean	PE	7.63
P_34	Pacific Ocean	PE	5.59
P_35	Pacific Ocean	PE	6.77
P_36	Pacific Ocean	PE	6.42
P_37	Pacific Ocean	PP	6.09
P_38	Pacific Ocean	PP	7.33
P_39	Pacific Ocean	Water	6.40
P_40	Pacific Ocean	Water	7.34
P_41	Pacific Ocean	Water	5.10
P_42	Pacific Ocean	Water	6.56
P_43	Pacific Ocean	Water	7.52
P_44	Pacific Ocean	Water	6.37
P_45	Pacific Ocean	Water	5.52
P_46	Pacific Ocean	Water	5.48
P_47	Pacific Ocean	Water	7.43
P_48	Pacific Ocean	Water	7.23

Supplementary Table S1. Contd.

P_49	Pacific Ocean	Water	6.78
P_50	Pacific Ocean	Water	6.76
P_51	Pacific Ocean	Water	7.71
P_52	Pacific Ocean	Water	7.30
A_1	Atlantic Ocean	HDPE	4.88
A_2	Atlantic Ocean	HDPE	6.57
A_3	Atlantic Ocean	HDPE	6.31
A_4	Atlantic Ocean	HDPE	8.09
A_5	Atlantic Ocean	HDPE	5.14
A_6	Atlantic Ocean	PP	6.40
A_7	Atlantic Ocean	HDPE	6.82
A_8	Atlantic Ocean	HDPE	7.26
A_9	Atlantic Ocean	HDPE	6.30
A_10	Atlantic Ocean	HDPE	7.67
A_11	Atlantic Ocean	HDPE	7.60
A_12	Atlantic Ocean	HDPE	7.02
A_13	Atlantic Ocean	HDPE	7.32
A_14	Atlantic Ocean	HDPE	7.30
A_15	Atlantic Ocean	HDPE	7.33
A_16	Atlantic Ocean	HDPE	7.62
A_17	Atlantic Ocean	HDPE	7.13
A_18	Atlantic Ocean	HDPE	6.74
A_19	Atlantic Ocean	PP	7.82
A_20	Atlantic Ocean	HDPE	8.75
A_21	Atlantic Ocean	PP	7.76
A_22	Atlantic Ocean	HDPE	5.27
A_23	Atlantic Ocean	PP	7.48
A_24	Atlantic Ocean	HDPE	6.06
A_25	Atlantic Ocean	PP	6.51
A_26	Atlantic Ocean	HDPE	7.60
A_27	Atlantic Ocean	HDPE	5.82
A_28	Atlantic Ocean	HDPE	7.01
A_29	Atlantic Ocean	HDPE	7.45
A_30	Atlantic Ocean	HDPE	7.43

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