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Synthetic mucus for an *ex vivo* phonation setup: Creation, application, and effect on excised porcine larynges

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ABSTRACT:

Laryngeal mucus hydrates and lubricates the deformable tissue of the vocal folds and acts as a boundary layer with the airflow from the lungs. However, the effects of the mucus' viscoelasticity on phonation remain widely unknown and mucus has not yet been established in experimental procedures of voice research. In this study, four synthetic mucus samples were created on the basis of xanthan with focus on physiological frequency-dependent viscoelastic properties, which cover viscosities and elasticities over 2 orders of magnitude. An established *ex vivo* experimental setup was expanded by a reproducible and controllable application method of synthetic mucus. The application method and the suitability of the synthetic mucus samples were successfully verified by fluorescence evidence on the vocal folds even after oscillation experiments. Subsequently, the impact of mucus viscoelasticity on the oscillatory dynamics of the vocal folds, the subglottal pressure, and acoustic signal was investigated with 24 porcine larynges (2304 datasets). Despite the large differences of viscoelasticity, the phonatory characteristics remained stable with only minor statistically significant differences. Overall, this study increased the level of realism in the experimental setup for replication of the phonatory process enabling further research on pathological mucus and exploration of therapeutic options. © 2022 Acoustical Society of America. https://doi.org/10.1121/10.0015364

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I. INTRODUCTION

Social interaction and numerous professions rely on a healthy voice. Severe voice disorders can interfere with social life and may lead to economical disadvantages.[1] Overall, dysphonic persons report a reduced quality of life.[2]

To overcome these drawbacks and improve the daily life of concerned individuals, treatment of voice and speech disorders is necessary. This requires an in-depth knowledge and understanding of phonation.

The principle of sound production in the larynx is known as fluid–structure–acoustic interaction. The airflow (fluid) from the lungs excites a self-sustained oscillation of the deformable tissue of the vocal folds (structure) and generates the primary sound signal of voice (acoustic). This sound is then modulated by the supraglottal structures in the vocal tract to generate articulated speech.

The interplay of the airflow with the tissue of the vocal folds is affected by the structural properties of the vocal folds, especially the mucosa, the outermost layer in the

three-layer scheme.[3] The mucosa is covered by the mucus, which was hypothesized to be a key element of the energy-transfer between the airflow and the tissue.[4] Mucus serves many tasks in the human body.[5] The air-exposed vocal folds are hydrated by the mucus and lubricated during vibration.[6] Up until now, the impact of mucus on the oscillation of the vocal folds and the resultant acoustics is not fully understood.

Mucus of varying thickness can be found for persons with and without voice disorders.[7,8] The consistency of mucus is affected by its composition and hydration, which can be altered by specific diseases, such as asthma, cystic fibrosis, active laryngeal tuberculosis, or chronic obstructive pulmonary disease, but also by simple inflammatory diseases of the airways.[9–11] Voice impairment has already been demonstrated for active laryngeal tuberculosis[11] and cystic fibrosis.[10] A reduced mucus production as caused by the disease ectodermal dysplasia[12] also has a negative impact on phonation.[13,14] Multiple studies regarding the effects of hydration and dehydration of the larynx on the acoustics have already been conducted.[15–18] As result, dehydration was found to affect acoustic voice parameters negatively.[19]

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However, research about the impact of mucus composition and rheology is rare. The first studies regarding the impact of different viscosities revealed an effect on the fundamental frequency of the oscillating vocal folds, the contact phase, and parameters based on the glottal area waveform (GAW). In these *ex vivo* studies with excised larynges, chondroitin sulfate sodium salt[20] and xanthan[21] were used as synthetic mucus substitutes. In another study, poly(styrene) sulfonate was used to mimic the interconnected mucins in a fundamental manner.[4] Mucins are the main ingredient of the water-based mucus and the primary determinant of its rheology.[22]

Recent research documented the physiological range of the rheology of human laryngeal mucus samples from the vocal folds of persons without voice disorders.[23] These findings enable the fabrication of a synthetic mucus with realistic viscoelastic properties. In combination with a defined application of mucus on the vocal folds, this enables to conduct reproducible and controllable *ex vivo* experimental investigations regarding the impact of mucus on phonation. *Ex vivo* setups are an established tool in voice research and offer automated measurements with excellent accessibility for pressure and acoustics measurements. Additionally, vocal fold oscillations can be captured easily by a high-speed camera.[24]

To the best of our knowledge, the impact of mucus viscoelasticity on phonation has not yet been investigated. In this study, the following goals were achieved:

(1) Creation of four synthetic mucus samples within the natural range of viscoelasticity based on the results of human laryngeal mucus rheology presented by Peters *et al.*[23]
(2) Expansion of the experimental setup developed by Birk *et al.*[24] by a reproducible, traceable, and controllable method of mucus application.
(3) Validation of the use of the synthetic mucus in *ex vivo* experiments in a preliminary study with 24 porcine larynges, revealing physiological phonation parameters for synthetic mucus in the physiological viscoelastic range.

The integration of synthetic mucus in the automated experimental setup enables a systematic investigation of the role of laryngeal mucus as a boundary layer in the phonation process as a part of the fluid–structure–acoustic interaction.

II. METHODS

A. Creation of synthetic mucus

1. Composition of synthetic mucus

In general, mucus consists of 1%–5% of mucins, 90%–95% water, 1% electrolytes, 1%–2% of lipids, deoxyribonucleic acid, proteins, cells, and cellular debris.[5,25] The main ingredient of mucus, the mucins, largely determine its rheology and are responsible for gelling. Mucins are polymeric glycoproteins that can build networks of different

strengths, depending on their content.[26] Porcine gastric mucins are commercially available but have lost their ability to undergo gelation as a result of the purification process.[27] Approaches to overcome this drawback by alternative procedures of mucin purification[28] or by chemical cross-linking of the denatured mucins[29,30] have already been developed. However, in this preliminary study, we decided to design the synthetic mucus as simple as possible and focused on the viscoelastic behavior rather than the exact composition.

To prevent degeneration by dehydration of the larynx, saline solution (with a physiological salt concentration of 0.9% as the standard in *ex vivo* experiments) was used in the experiments as a reference (S). It was also used as basis for the creation of synthetic mucus.

For synthetic mucus creation, xanthan was chosen as gelling agent to replicate the viscoelasticity of physiological mucus. It was chosen due to its widespread use in the pharmaceutical and food industry.[31,32] This makes it a potential candidate for the development of synthetic mucus for therapeutic strategies. Moreover, xanthan solutions showed in our preliminary investigations highly adaptable viscoelastic properties and the viscoelasticity was stable against the addition of salts for generation of physiological salt concentration and stable against the addition of mucins.

Three of the four synthetic mucus samples were synthesized by adding xanthan (extra pure, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) to saline solution to different contents (mass percentage: mass solute / mass total solution) of 0.1% (M1), 0.25% (M2), and 0.75% (M4) under magnetic stirring. The solutions were stirred until all xanthan was completely dissolved. Next, the synthetic mucus samples were placed into a rotary mixer and mixed carefully overnight for formation of a homogenous gel. Another synthetic mucus sample (M3) was created by 0.5% mucins (type III, bound sialic acid 0.5%–1.5%, partially purified powder, Sigma-Aldrich, St. Louis, MO) in addition to 0.25% xanthan. The mucins were added prior to xanthan. The aqueous mucin solution was stirred until the mucins were completely dissolved. Then it was mixed carefully overnight by a rotary mixer in the fridge. The next day, xanthan was added following the same procedure as described for the other synthetic mucus samples. The synthetic mucus samples were stored in a fridge at 5 °C and used in the *ex vivo* experiments within 5 days.

2. Rheological analysis

The rheological investigations of synthetic mucus were performed by passive particle tracking microrheology (PTM). This technique was used for comparability reasons with previous investigations regarding the viscoelasticity of human laryngeal mucus of the vocal folds. Except for the camera, the setup was the same as presented in Peters *et al.*[23] It was developed by the Biophysics Group, Department of Physics, FAU Erlangen-Nürnberg. FluoSpheres™ carboxylate-modified microspheres (Thermo Fisher Scientific, Waltham, MA), orange fluorescent (540 nm/560 nm) with a diameter of 1 μm were used. In addition, 0.5 μl of the 1% microsphere solution

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(diluted from 2%) were mixed with $20\,\mu l$ synthetic mucus. The mixture was sealed by a Gene Frame (Thermo Fisher Scientific, Waltham, MA) between a glass slide and a coverslip (0.25 mm gap width) and placed onto a $100\times$ magnification CFI Plan Apochromat microscopy objective lens (Nikon, Minato, Japan). The fluorescent microspheres were excited by an MGL-H-532 1 W laser (Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, P.R. China) with a wavelength of 532 nm. The camera was a Basler acA20-520 (Basler AG, 22 926 Ahrensburg, Germany) and recorded the microspheres' movements with a frame rate of 500 frames per second (fps). The measurements were executed at a room temperature of 25 °C. One hundred microspheres were tracked in each synthetic mucus sample and their mean square displacements (MSD) were determined. In the next step, the median was applied to the MSDs of all 100 microspheres and the diffusive exponent α was determined followed by the calculation of the frequency-dependent viscoelasticity (storage modulus G', loss modulus G''). The calculation was based on a procedure of Mason.[33] More detailed information about the setup and the evaluation can be found in Peters et al.[23]

B. Experimental setup

1. Porcine cadaver larynges

The 24 porcine larynges (L1–L24) were provided by a local slaughterhouse. A few hours postmortem, the larynges were quick-frozen with 2-methylbutan (–150 °C) and stored at –80 °C. At this temperature, the tissue properties are preserved.[34] Before the experiments, the larynges were slowly thawed in a refrigerator and then soaked in saline solution. The supraglottal tissue cranial to the false vocal folds was removed. As consequence, the passages for secretion towards the ventricles were capped. The leakages were closed with Histoacryl® (B. Braun SE, Melsungen, Germany) tissue glue. A plane was generated on the upper regions of the arytenoid cartilages for mounting of the prongs of the arytenoid cartilage manipulators. These induced a torque on the arytenoid cartilages and an adduction of the vocal folds. Additionally, a surgical suture was sewn into the tip of the thyroid cartilage for attachment of a weight. The weight tilts the thyroid cartilage and elongates the vocal folds, which simulates the contraction of the cricothyroid muscle.

2. Automated measurement setup

The experimental procedure is based on a well-established ex vivo experimental setup introduced by Birk et al.,[24] which was also utilized in further studies.[35,36] The setup is shown in Fig. 1(a). The trachea of the larynx was mounted on a steel tube with a diameter of 20 mm. In the subglottal region, the mass flow Q was adjusted by a 1579 A/B (MKS, Andover, MA) mass flow controller and a 4000B (MKS) digital power supply. Tissue dehydration was avoided through use of an ultrasound nebulizer Ultrasonat 810 (Hico, Hirtz & Co. KG, Köln, Germany) which regulated temperature and moisture and was integrated into the air supply. A XCS-93-5PSISG pressure sensor (Kulite Semiconductor Products, Inc., Leonia, NJ) was placed 130 mm below the glottal plane of the larynx for time resolved recording of the subglottal pressure P_{sub}. The sensor was driven by a PXIe-4330 (National Instruments, Austin, TX) bridge module and it was sampled for 2 s with a sampling rate of 96 kHz. The oscillation patterns of the vocal
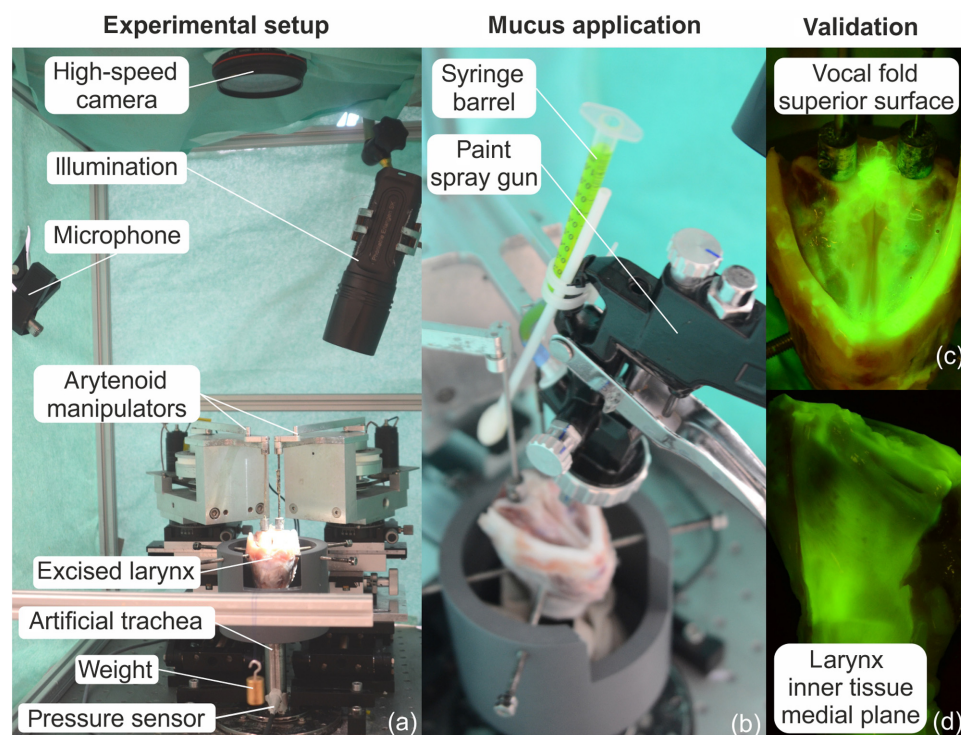

FIG. 1. (Color online) (a) Experimental setup for ex vivo experiments, introduced by Birk et al. (Ref. 24). (b) Expansion of the setup by a method for synthetic mucus application by a modified paint spray gun. (c) Fluorescent vocal fold superior surface and (d) inner tissue of the larynx at the medial plane after spraying fluorescent synthetic mucus on top of the vocal folds and through the glottis and performing oscillation experiments.

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and ventricular folds were recorded for 0.5 s by a Phantom V2511 (Vision Research, Wayne, NJ) high-speed camera with an EF180 mm f/3.5 macro lens (Canon, Inc., Tokyo, Japan) at a frame rate of 4 kHz and a spatial resolution of 768×768 pixels. The resulting acoustic signal was recorded with a sampling rate of 96 kHz for a duration of 2 s by a 4189 (Brüel & Kjær, 2850 Nærum, Denmark) $1/2$ in. free-field microphone. The microphone was mounted in the coronal plane of the larynx at a distance of 30 cm downstream from the glottis, with an inclination of $45°$ relative to a horizontal plane. A Nexus 2690 microphone conditioning amplifier (Brüel & Kjær) amplified the acoustic signal before it was sampled by a 4492 (National Instruments) dynamic signal acquisition module. The setup was controlled by a PC and LabView (National Instruments). The initial vocal fold elongation was achieved by a weight of 20 g that was attached to a surgical suture that was sewn into the tip of the thyroid cartilage. Vocal fold adduction was achieved by two electro-mechanic devices which induced an independent torque (T [mNm]) on the left (T_L) and right (T_R) arytenoid cartilage. Each was measured by a TD70 (ME Meßsysteme GmbH, Henningsdorf, Germany) sensor. A prephonatory gap was induced by a plastic shim of 2 mm thickness, which was placed in the posterior region of the larynx between the arytenoid cartilages. More detailed information about the experimental setup is given by Birk et al.[24] Saline solution was applied with a soaked pad. For mucus application, the setup was extended by a modified paint spray gun (FERM, Zwolle, The Netherlands) [Fig. 1(b)]. The paint container was replaced by the barrel of a 1 ml syringe, to spray defined amounts of mucus. Rheological measurements before and after the spraying process showed that the spraying of the synthetic mucus did not have a distinct impact on its viscoelasticity. The mucus samples were sprayed onto the superior surface of the vocal folds and through the glottis into the larynx. For the visualisation of the distribution of sprayed synthetic mucus, proof of bioadhesion, and validation of mucus addition, the synthetic mucus samples were mixed with Fluorescein Alcon® 10% (Novartis AG, Basel, Switzerland) to a final fluorescein concentration of 0.01‰ for preliminary tests. As shown in Fig. 1(c), the superior surface of the vocal folds and the inner tissue of the larynx in the sagittal plane, Fig. 1(d), were fluorescent after spraying and after performing oscillation experiments.

3. Measurement procedure

An overview of the measurement procedure for each porcine larynx is given in Table I. Each of the four synthetic mucus samples was applied to six larynges. For each larynx, the measurement series was first performed with the saline solution as reference and then repeated with application of synthetic mucus. A measurement series consisted of the phonation onset and an increase in glottal airflow five times with steps of 10 standard liters/min (SLM). Phonation onset was determined subjectively by increasing the airflow until a self-sustained phonation of the vocal folds was reached. Saline solution or synthetic mucus was applied before every measurement series

TABLE I. Measurement protocol for each of the porcine larynges. Each of the four synthetic mucus samples (M1–M4) was applied to six larynges. A total of 96 measurement runs were conducted for each larynx which resulted in 2304 measurement runs. The measurement runs were performed according to the table from top to bottom.

Vocal fold hydration	Glottal gap	Adduction T_L : T_R (mNm)	Airflow
Saline solution (S)	No gap	$A_1 = 10{:}10$	Onset $+ 5 \times 10$ SLM
		$A_2 = 10{:}15$	Onset $+ 5 \times 10$ SLM
		$A_3 = 10{:}20$	Onset $+ 5 \times 10$ SLM
		$A_4 = 20{:}20$	Onset $+ 5 \times 10$ SLM
	Gap	$A_1 = 10{:}10$	Onset $+ 5 \times 10$ SLM
		$A_2 = 10{:}15$	Onset $+ 5 \times 10$ SLM
		$A_3 = 10{:}20$	Onset $+ 5 \times 10$ SLM
		$A_4 = 20{:}20$	Onset $+ 5 \times 10$ SLM
Synthetic mucus (M) M1: 0.10% xanthan M2: 0.25% xanthan M3: 0.25% xanthan, 0.50% mucin M4: 0.75% xanthan	Gap	$A_1 = 10{:}10$	Onset $+ 5 \times 10$ SLM
		$A_2 = 10{:}15$	Onset $+ 5 \times 10$ SLM
		$A_3 = 10{:}20$	Onset $+ 5 \times 10$ SLM
		$A_4 = 20{:}20$	Onset $+ 5 \times 10$ SLM
	No gap	$A_1 = 10{:}10$	Onset $+ 5 \times 10$ SLM
		$A_2 = 10{:}15$	Onset $+ 5 \times 10$ SLM
		$A_3 = 10{:}20$	Onset $+ 5 \times 10$ SLM
		$A_4 = 20{:}20$	Onset $+ 5 \times 10$ SLM

on the larynx. The amount of sprayed synthetic mucus was kept constant at 0.5 ml. The measurement series was performed with and without pre-phonatory glottal gap and four symmetric and asymmetric vocal fold adduction levels A_i of the left and right vocal fold, induced by the arytenoid cartilage manipulators ($A_i = T_L$ [mNm]: T_R [mNm]): $A_1 = 10{:}10$, $A_2 = 10{:}15$, $A_3 = 10{:}20$ and $A_4 = 20{:}20$. Glottal closure and vocal fold adduction were manipulated to generate a large variety of vibration patterns and acoustic outcomes. This resulted in 96 measurement runs for each larynx and 2304 datasets in total: 2 (saline solution vs mucus) $\times$ 2 (no gap vs gap) $\times$ 4 (adduction levels) $\times$ 6 (flow steps) $\times$ 24 (larynges) = 2304.

C. Data analysis

The computed parameters were divided into four groups: the aerodynamic parameters, the glottal dynamic parameters of the high-speed imaging (HSI) of the vocal folds, the subglottal pressure parameters (P_{sub}), and the acoustic parameters (audio), which are well established in voice research.[37,38] The aerodynamic parameters were computed by MATLAB (The MathWorks, Inc., Natick, MA), the other parameters were evaluated using Glottis Analysis Tools 2020 (GAT),[39,40] a software package developed in-house. Table II provides an overview and a short description of the computed parameters. More detailed information is given in the references in Table II.

1. Aerodynamic parameters

The glottal flow resistance R_B is a measure of phonation efficiency. It describes the energy transfer between glottal

TABLE II. Parameters evaluated in this study. (a) Aerodynamic parameters. (b) Glottal dynamic parameters. (c) Subglottal pressure and acoustic parameters.

Abbreviation	Parameter	Description
(a) Aerodynamic parameters		
R_B (Pa/SLM)	Glottal flow resistance[41]	The higher, the better
		$\bar{P}_{sub}(t)/\bar{Q}$
SPL (dB)	Sound pressure level[42]	The higher, the louder
(b) Glottal dynamic parameters		
GGI (AU)	Glottis Gap Index[43]	0–1
		Minimum glottal area/ maximum glottal area
OQ (AU)	Open quotient[44]	0–1
		Open time of glottis/ cycle duration
CQ (AU)	Closing quotient[45]	0–1
		Closing time of glottis/ cycle duration
AP (AU)	Amplitude periodicity[46]	0–1
		1: Identical amplitude sizes
TP (AU)	Time periodicity[46]	0–1
		1: Identical cycle lengths
PAI (AU)	Phase asymmetry index[46]	0–1
		0: GAW cycles (left and right) are in phase
ASI (AU)	Amplitude symmetry index[47]	0–1
		1: GAW cycles (left and right) are even in size
(c) Subglottal pressure and acoustic parameters		
HNR (dB)	Harmonics to noise ratio[48]	The higher, the better
CPP (dB)	Cepstral peak prominence[49]	The higher, the better
Shim (%)	Shimmer[50]	The smaller, the better
Jitt (%)	Jitter[50]	The smaller, the better

flow and oscillating tissue and is the quotient of the measured glottal pressure difference and the average of the applied airflow $\bar{Q}$.[41] In the absence of a vocal tract, the glottal pressure difference is the temporal average of the subglottal pressure signal $\bar{P}_{sub}(t)$, which is computed from the time-resolved pressure signal. The time-resolved acoustic signal leads to calculation of the sound pressure level (SPL). The aerodynamic parameters are summarized in Table II(a).

2. Glottal dynamic parameters

For determination of the fundamental frequency of the vocal fold oscillation $F_{0,HSI}$ and glottal dynamic parameters [Table II(b)], the high-speed videos were analyzed with GAT.[39] The GAW was determined by segmentation of the high-speed videos prior to calculation of vocal fold dynamics.[40] The glottal dynamics are described by parameters of glottal closure [glottis gap index (GGI), open quotient (OQ),

closing quotient (CQ)]; periodicity [amplitude periodicity (AP), time periodicity (TP)]; and symmetry [amplitude symmetry index (ASI), phase asymmetry index (PAI)]. The analysis of the GAWs were performed on a sequence of 20 consecutive cycles which was the highest common number of oscillation cycles in the recordings. This is in accordance with the minimum number of cycles suggested for a reliable analysis.[51]

3. Subglottal pressure and acoustic parameters

GAT was also used to analyze the time-resolved subglottal pressure and acoustic signal. The full length of the signals (2 s) was analyzed to reach the recommended minimum number of cycles, i.e., 100.[52,53] The analysis included cycle detection, determination of the fundamental frequency ($F_{0,Psub}$, $F_{0,audio}$), and calculation of parameters to access information about signal quality. These parameters revealed noise [harmonics to noise ratio (HNR)] and regularity [jitter (Jitt), shimmer (Shim), cepstral peak prominence (CPP)] [Table II(c)].

D. Statistical analysis

The statistics were performed with IBM SPSS version 26 (IBM, Armonk, NY). The parameters were compared with regard to the saline solution (S) and synthetic mucus samples (M1–M4) that were applied on the vocal folds. First, a Kolmogorov–Smirnov test was performed to check on the normal distribution of the data. Only HNR_{audio} was normally distributed. Hence, non-parametric tests were used for mean comparison of all parameters. For group comparisons, the Kruskal–Wallis test was calculated with a significance level of p = 0.05. Subsequent *post hoc* Mann–Whitney U tests with a Bonferroni correction were performed for significant cases. The correction factor was chosen in accordance with the number of tests, i.e., n = 10, and resulted in a significance level of p = 0.005 (= 0.05/10). The statistical analysis is conceptualized to answer the following questions:

- Question 1: How does synthetic mucus and its viscoelasticity influence the dynamics of the vibratory vocal folds?
- Question 2: What impact does synthetic mucus and viscoelasticity have on the subglottal pressure and acoustic parameters?
- Question 3: Do mucins have an impact on glottal dynamic, subglottal pressure or acoustic parameters?

III. RESULTS

A. Creation of synthetic mucus

The rheology of human laryngeal mucus from the vocal folds of vocally healthy subjects was investigated in a previous study.[23] Due to the limited sample amount, the viscoelasticity was determined by PTM. The 19 investigated mucus samples revealed a diverse viscoelasticity, but the data could be grouped into three mucus types with similar

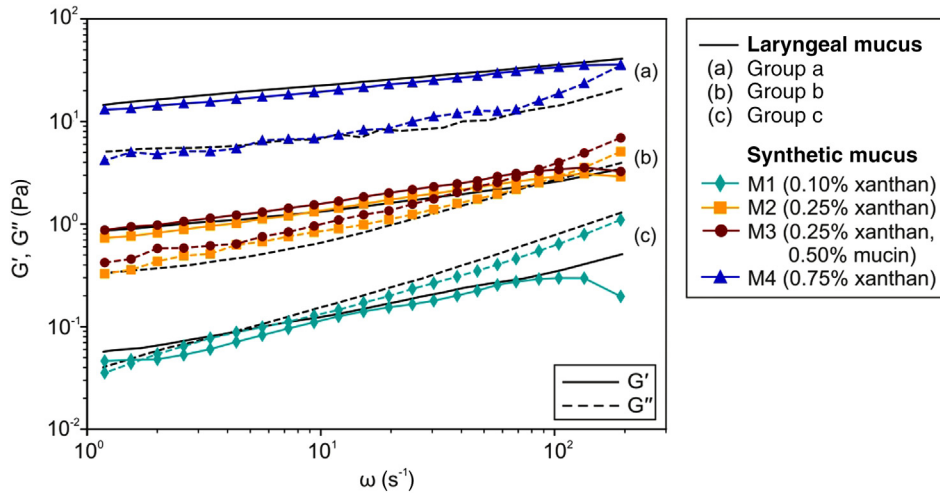J. Acoust. Soc. Am. **152** (6), December 2022

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FIG. 2. (Color online) (a)–(c) Mean viscoelasticities of the three groups of human laryngeal mucus (black lines) collected from the vocal folds, determined by Peters et al. (Ref. 23) in comparison with xanthan solutions of different concentrations (M1, M2, and M4) and with the addition of mucin (M3).

rheological characteristics. The mean viscoelasticities for the three resulting groups are shown in Fig. 2, indicated by the black continuous (storage modulus G') and dashed (loss modulus G'') lines. Mucus of Group a revealed solid-like, gel character and the highest rigidity. Mucus of Group b showed predominantly solid-like viscoelasticity and a crossover of G' and G'' at high frequencies. Mucus of Group c revealed a crossover of G' and G'' at low frequencies, predominantly liquid-like viscoelasticity, and the smallest rigidity.

The rheology of xanthan solutions of different concentrations was investigated by PTM. It was found that xanthan solutions revealed viscoelasticity similar to human laryngeal mucus. The rigidity of the xanthan solutions could be easily adjusted by the xanthan content which enabled the replication of the viscoelasticity of human laryngeal mucus. As shown in Fig. 2, the viscoelasticity of 0.10% xanthan (M1) is similar to mucus of Group c. The absolute G' and G'' were in the same range and the crossover of G' and G'' for both was between $\omega = 1\,s^{-1}$ and $\omega = 10\,s^{-1}$. An increase in xanthan concentration increased the absolute values of G' and G'' and shifted the crossover to higher frequencies. A concentration of 0.25% xanthan (M2) was similar to mucus of Group b. The absolute values of G' and G'' were similar and the crossover for both was found to be approximately $\omega = 100\,s^{-1}$. Finally, a further increase in xanthan concentration to 0.75% (M4) led to a viscoelasticity similar to mucus of Group a. The absolute values of G' and G'' were in the same range. However, a convergence of G' and G'' was observed for xanthan at about $\omega = 200\,s^{-1}$ whereas it was not so for the human mucus. The addition of 0.50% mucin

to 0.25% xanthan (M3) revealed a slight increase in G' and G'' and a slight decrease in crossover frequency compared to M2.

The G' and G'' of the synthetic mucus samples covered a large range of viscoelasticity. M1 revealed at the lower evaluation limit ($\omega = 1.2\,s^{-1}$) viscoelastic moduli of $G' = 0.046\,\mathrm{Pa}$ and $G'' = 0.035\,\mathrm{Pa}$. M4 revealed at the lower evaluation limit $G' = 13.04\,\mathrm{Pa}$ and $G'' = 4.18\,\mathrm{Pa}$. Hence, the factor between M1 and M4 is 284 for G' and 119 for G''. At the higher evaluation limit ($\omega = 191.9\,s^{-1}$), M1 showed $G' = 0.20\,\mathrm{Pa}$ and $G'' = 1.10\,\mathrm{Pa}$ and M4 revealed $G' = 35.91\,\mathrm{Pa}$ and $G'' = 35.30\,\mathrm{Pa}$. Hence, the factor between M1 and M4 is 180 for G' and 32 for G'' at the higher evaluation limit.

B. General phonation parameters

Based on the GAW signal, only datasets of regular and periodic oscillations, which were classified as type 1 signals by Titze[54] were considered in this study. Periodic oscillations are assumed for healthy voice production;[37] hence, type 1 signals can be related to non-disordered, normal oscillations. Measurement runs with other oscillation patterns were excluded from evaluation. This led to a total of 1660 datasets. The range and mean values of the general phonation parameters are given in Table III for phonation onset and overall measurements. The fundamental oscillation frequency of the vocal folds $F_{0,HSI}$ matched the fundamental frequency of the acoustic signal $F_{0,audio}$ and the subglottal pressure $F_{0,Psub}$ for phonation onset and all recordings. The standard deviations of the flow rate $\bar{Q}$,

TABLE III. Mean and standard deviation values of the general phonation parameters $F_{0,HSI}$, $F_{0,audio}$, $F_{0,Psub}$, $\bar{P}_{sub}$, $\bar{Q}$, R_B, and SPL for phonation onset and over all recordings and minimum and maximum values over all recordings.

	$F_{0,HSI}$ (Hz)	$F_{0,audio}$ (Hz)	$F_{0,Psub}$ (Hz)	$\bar{P}_{sub}$ (Pa)	$\bar{Q}$ (SLM)	R_B (Pa/SLM)	SPL (dB)
Phonation onset (N = 277)	76 ± 13	76 ± 13	76 ± 13	679 ± 236	26 ± 18	41.9 ± 30.9	72.9 ± 4.7
All recordings (N = 1660)	89 ± 15	89 ± 15	89 ± 15	1398 ± 710	50 ± 24	33.9 ± 20.5	81.5 ± 6.2
Minimum	42	42	42	214	6	5.5	62.4
Maximum	140	140	139	4196	120	188.9	96.4

subglottal pressure $\bar{P}_{sub}$, and R_B were high, due to the inclusion of measurement series with and without induced prephonatory gap, the individuality of the larynges, and the flow steps within the measurement series.

In Fig. 3, the distribution of $\bar{Q}$ (a), the SPL (b), and $F_{0,audio}$ (c) are visualized for each larynx and measurement run. The parameters showed a homogenous distribution for all larynges and no outliers were displayed. $\bar{Q}$ and $F_{0,audio}$ showed linear trends whereas the SPL revealed a logarithmic trend with respect to the subglottal pressure $\bar{P}_{sub}$.

C. Impact of synthetic mucus on phonation

1. Glottal dynamic parameters

Question 1: How does synthetic mucus and viscoelasticity influence the dynamics of the vibratory vocal folds?

The impact of the synthetic mucus and viscoelasticity on the glottal dynamic parameters is visualized by box plots in Fig. 4. The corresponding median and additionally mean values are given in Table IV.

GGI, *OQ*, and *CQ* are measures of glottal closure. As shown in Fig. 4(a), the *GGI* for saline solution (S) revealed more outliers than the synthetic mucus samples M1–M4. This was due to the larger number of runs performed with S and can be also seen for all other glottal dynamic parameters. The distribution of *GGI* was the narrowest for M2. This is also valid for the other parameters except *ASI* [Fig. 4(e)]. The median *GGI* was zero for S, M2, and M3 in contrast to M1 and M3. This can be explained by the unbalanced number of runs with and without induced prephonatory glottal gap due to exclusion of measurement data based on the oscillation type as described in the beginning of Sec. III B. This affected the median of the *GGI*, which is mostly zero without induced pre-phonatory gap. Hence, the median values of the *GGI* have to be interpreted with caution and the mean values are preferable for interpretation. The mean values of the *GGI* did not differ much

from each other and no trend was found for the samples from S to M4.

As already explained for the *GGI*, the median values of *OQ* also have to be interpreted with caution due to unbalanced measurement runs. The mean values of *OQ* did not differ much between the different mucus samples (Table IV). However, a slight decrease was found for the mucus samples with increasing xanthan concentration from M1 to M4.

The median values of *CQ* [Fig. 4(b)] increased slightly from S to M1. The median of M1 was in the same range as M2 and decreased again over M3 to M4. However, the differences were very small.

AP and *TP* describe the periodicity of the GAW signal and are shown in Figs. 4(c) and 4(d). The differences of these two parameters were very small between the mucus samples, which is emphasized by the exact values in Table IV.

The *ASI* [Fig. 4(e)] and the *PAI* [Fig. 4(f)], are measures of the spatial and temporal symmetry between the left and right GAWs. The median of the ASI was the highest for M2 measurements. It was lower and at about the same level for the other samples (Table IV). The mean values revealed a slight decrease from S to M4 with the exception of M2.

The median values of the *PAI* were similar for the synthetic mucus samples except for M4. A difference between the samples was found regarding the distribution, which was wider for M3 and M4 than for the others.

The Kruskal–Wallis test between saline solution and the synthetic mucus samples was significant for all glottal dynamic parameters except for *OQ* [Table VII(a)]. The subsequent *post hoc* tests (Mann–Whitney U) showed statistically significant differences in some comparisons. None of the pairwise comparisons between saline solution and the synthetic mucus samples revealed statistically significant differences among all parameters. Statistically significant differences between saline and the synthetic mucus samples were found between S and M1 for the $F_{0,HSI}$, *AP*, and *TP*, between S and M2 for the *ASI* and between S and M3 for the *AP*. The largest number of statistically significant
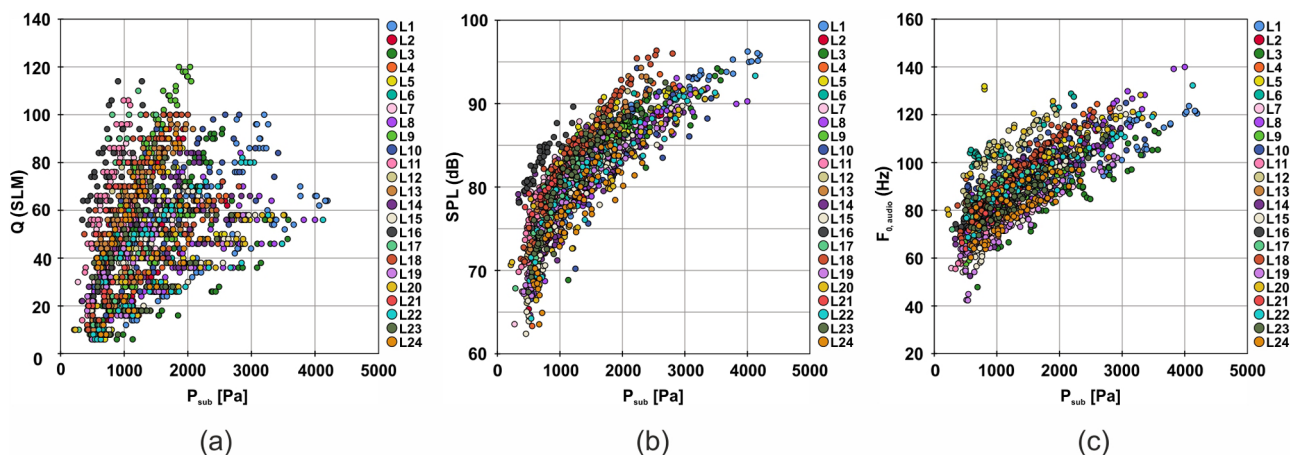

FIG. 3. (Color online) General phonation parameters over the subglottal pressure $\bar{P}_{sub}$ for all larynges L1–L24 and measurement runs: (a) $\bar{Q}$, (b) SPL, and (c) $F_{0,audio}$.

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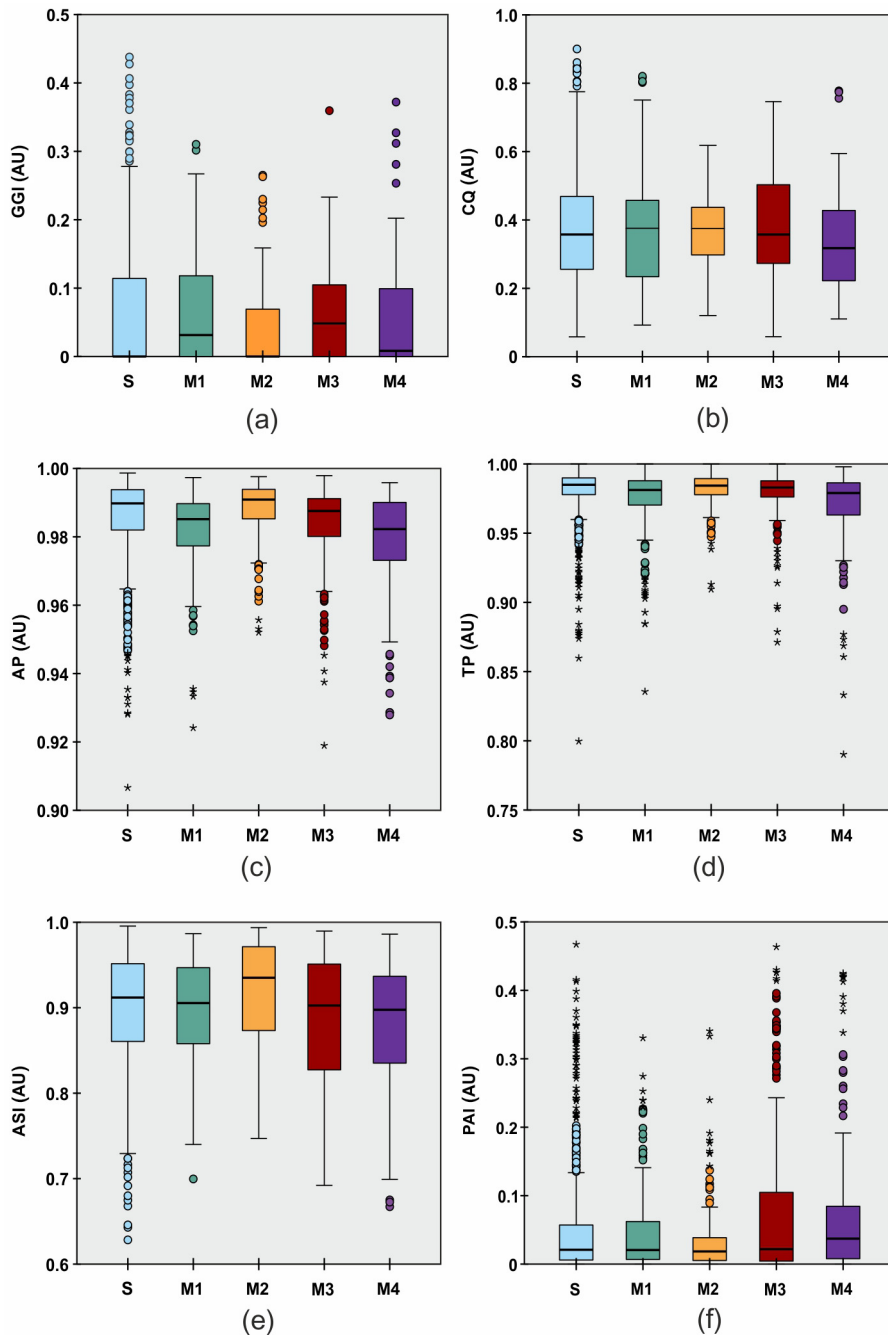
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FIG. 4. (Color online) Box plots of the glottal dynamic parameters of saline solution (S) and the synthetic mucus samples (M1–M4): (a) *GGI*, (b) *CQ*, (c) *AP*, (d) *TP*, (e) *ASI*, and (f) *PAI*.

differences between saline solution and the synthetic mucus samples were found between S and M4, which are the $F_{0,HSI}$, *CQ*, *AP*, *TP*, and *ASI*. The comparison of the synthetic mucus samples showed statistically significant differences between M1 and M2 of $F_{0,HSI}$, *GGI*, *AP*, *TP*, and *ASI*, but between M1 and M3 and between M1 and M4 only for the $F_{0,HSI}$. *GGI*, *AP*, and *ASI* were statistically significantly different between M2 and M3. The most statistically significant differences between the synthetic mucus samples were found between M2 and M4. There, all parameters revealed statistically significant differences with exception of the *GGI* and $F_{0,HSI}$. Between M3 and M4, $F_{0,HSI}$, *CQ*, *AP*, and *TP* showed statistically significant differences.

2. Subglottal pressure and acoustic parameters

Question 2: What impact does synthetic mucus and viscoelasticity have on the subglottal pressure and acoustic parameters?

Mean values and median values of the subglottal pressure parameters are given in Table V for saline solution and each synthetic mucus sample. The absolute differences between the groups of each subglottal pressure parameter were small.

The results of the statistical analysis of the subglottal pressure parameters are given in Table VII(b). The Kruskal–Wallis test revealed statistically significant differences between the groups for all subglottal pressure parameters. The *post hoc* test (Mann–Whitney U) showed statistically

TABLE IV. Median and mean values with standard deviation of the $F_{0,HSI}$ and the glottal dynamic parameters GGI, OQ, CQ, AP, TP, PAI, and ASI for saline solution (S) and the synthetic mucus samples (M1–M4).

		$F_{0,HSI}$ (Hz)	GGI (AU)	OQ (AU)	CQ (AU)	AP (AU)	TP (AU)	PAI (AU)	ASI (AU)
S	Median	89	0.00	0.89	0.36	0.99	0.98	0.02	0.91
(N = 893)	Mean	89 ± 15	0.06 ± 0.08	0.75 ± 0.28	0.37 ± 0.15	0.99 ± 0.01	0.98 ± 0.02	0.05 ± 0.07	0.90 ± 0.07
M1	Median	92	0.03	1.00	0.38	0.99	0.98	0.02	0.91
(N = 191)	Mean	94 ± 14	0.06 ± 0.08	0.78 ± 0.29	0.37 ± 0.16	0.98 ± 0.01	0.97 ± 0.03	0.05 ± 0.07	0.90 ± 0.06
M2	Median	87	0.00	0.81	0.38	0.99	0.98	0.02	0.94
(N = 202)	Mean	87 ± 16	0.04 ± 0.06	0.77 ± 0.24	0.37 ± 0.10	0.99 ± 0.01	0.98 ± 0.01	0.03 ± 0.05	0.92 ± 0.06
M3	Median	89	0.05	1.00	0.36	0.99	0.98	0.02	0.90
(N = 219)	Mean	88 ± 13	0.06 ± 0.07	0.75 ± 0.28	0.38 ± 0.14	0.98 ± 0.01	0.98 ± 0.02	0.09 ± 0.13	0.89 ± 0.07
M4	Median	82	0.01	1.00	0.32	0.98	0.98	0.04	0.90
(N = 155)	Mean	84 ± 16	0.05 ± 0.08	0.72 ± 0.31	0.33 ± 0.13	0.98 ± 0.02	0.97 ± 0.03	0.08 ± 0.11	0.88 ± 0.07

significant differences between S and M1 for all subglottal pressure parameters, except CPP_{Psub}. No statistically significant differences were found between S and M2, and between S and M3 statistically significant differences were found for HNR_{Psub} and $Jitt_{Psub}$. Most of the statistically significant differences were found between S and M4 which includes all parameters. Within the groups of synthetic mucus samples, M1 and M2 showed statistically significant differences for $F_{0,Psub}$, HNR_{Psub}, and $Shim_{Psub}$. No statistically significant differences were found between M1 and M3 and also between M1 and M4 with the exception of the $F_{0,Psub}$. Between M2 and M3, statistically significant differences were found for HNR_{Psub} and $Jitt_{Psub}$ and between M2 and M4, $Shim_{Psub}$ was additionally significant. No statistically significant differences were found between M3 and M4 except for the $F_{0,Psub}$.

Figure 5 shows box plots of the evaluated acoustic parameters. The associated median and mean values are given in Table VI. In general, the absolute differences were small.

The CPP_{audio} is shown in Fig. 5(a). Saline solution revealed the most outliers which could be also observed for the $Jitt_{audio}$ and $Shim_{audio}$. The highest median HNR_{audio}, depicted in Fig. 5(b), was found for M2, the lowest was found for M3.

M3 revealed the highest median $Jitt_{audio}$ in Fig. 5(c) and also the widest distribution. Regarding $Shim_{audio}$ [Fig. 5(d)], the median values of S and M2 were at a similar level and were at a higher level for M1, M3, and M4.

The Kruskal–Wallis test [given in Table VII(c)] revealed statistically significant differences between the groups for each evaluated acoustic parameter. The subsequent *post hoc* tests (Mann–Whitney U) revealed inconsistent statistically significant differences among the group comparisons. The tests between saline solution and the synthetic mucus samples revealed statistically significant differences between S and M1 of $F_{0,audio}$, CPP_{audio}, and $Shim_{audio}$ and no differences between S and M2. Between S and M3, HNR_{audio}, $Jitt_{audio}$, and $Shim_{audio}$ revealed statistically significant differences. Most of the statistically significant differences were found between S and M4 and between M1 and M2 for all parameters except $Jitt_{audio}$. No statistically significant differences were found between M1 and M3 except for $F_{0,audio}$, which was the same between M1 and M4. Between M2 and M3 and also between M2 and M4, HNR_{audio} and $Shim_{audio}$ displayed statistically significant differences. No statistically significant differences were found between M3 and M4, except for the $F_{0,audio}$.

TABLE V. Median and mean values with standard deviation of the $F_{0,Psub}$ and the subglottal pressure parameters CPP_{Psub}, HNR_{Psub}, $Shim_{Psub}$, and $Jitt_{Psub}$ for saline solution (S) and the synthetic mucus samples (M1–M4).

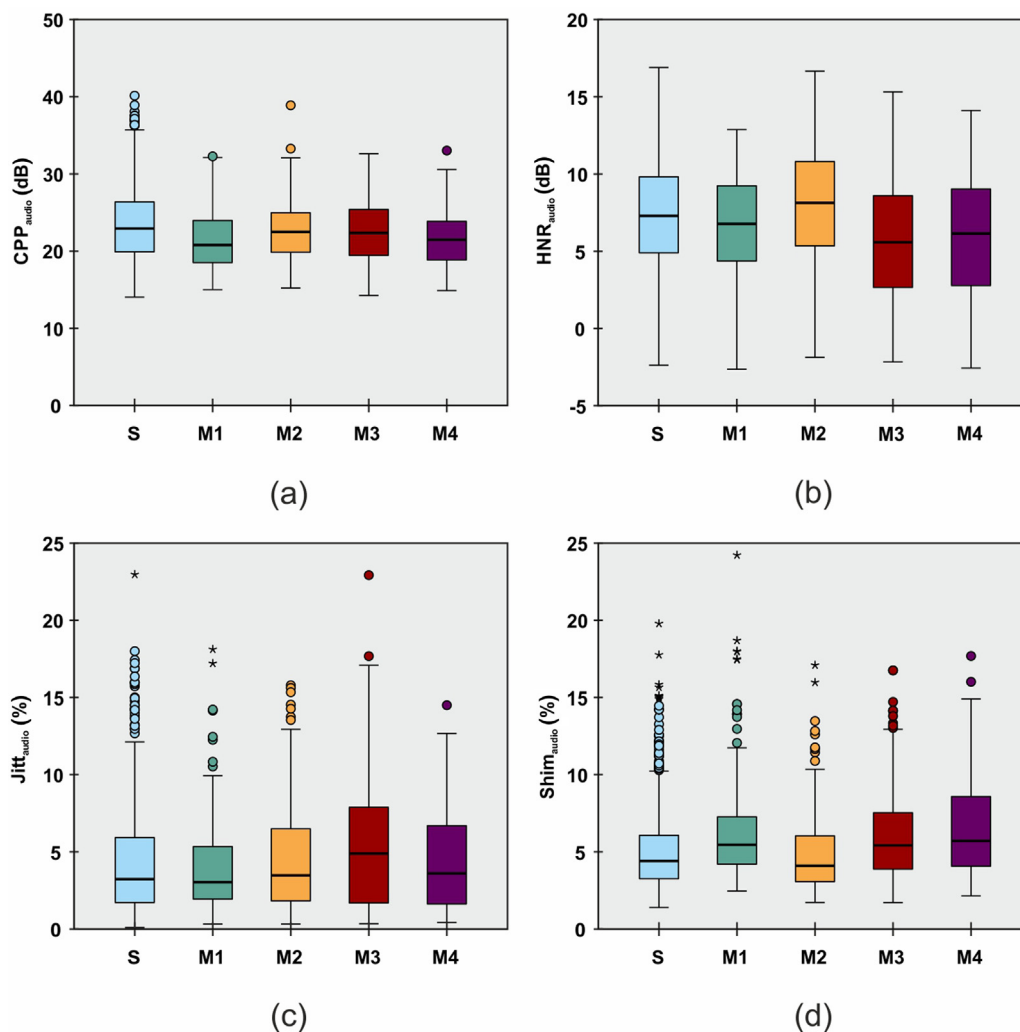|		$F_{0,Psub}$ (Hz)	CPP_{Psub} (dB)	HNR_{Psub} (dB)	$Shim_{Psub}$ (%)	$Jitt_{Psub}$ (%)
S	Median	89	20.8	15.0	2.0	2.2
(N = 893)	Mean	90 ± 15	21.6 ± 4.6	13.9 ± 4.9	2.9 ± 2.6	3.2 ± 3.1
M1	Median	92	20.0	12.8	2.7	2.9
(N = 191)	Mean	94 ± 14	20.8 ± 4.0	11.0 ± 5.5	3.6 ± 2.7	4.6 ± 4.4
M2	Median	87	20.4	15.7	2.1	2.1
(N = 202)	Mean	87 ± 16	20.5 ± 4.0	14.1 ± 5.1	2.7 ± 2.4	3.6 ± 3.6
M3	Median	89	20.2	13.6	2.3	2.7
(N = 219)	Mean	88 ± 13	20.7 ± 4.0	12.1 ± 5.5	3.4 ± 2.9	4.6 ± 4.1
M4	Median	81	19.7	12.5	3.0	3.0
(N = 155)	Mean	84 ± 16	19.6 ± 3.0	11.8 ± 5.5	4.1 ± 3.3	4.0 ± 3.3

FIG. 5. (Color online) Box plots of the acoustic parameters for saline solution (S) and the synthetic mucus samples (M1–M4): (a) CPP_{audio}, (b) HNR_{audio}, (c) $Jitt_{audio}$, and (d) $Shim_{audio}$.

Question 3: Do mucins have an impact on glottal dynamic, subglottal pressure, or acoustic parameters?

The impact of mucins can be analyzed by comparison of M2 with M3 since both samples contain the same amount of xanthan but differ in the addition of mucins.

M3 showed a wider distribution of the glottal dynamic parameters in Fig. 4. Statistically significant differences between M2 and M3 were found for *GGI*, *AP*, and *ASI* [Table VII(a)]. However, the absolute differences of the mean and median values (Table IV) were small.

TABLE VI. Median and mean values with standard deviation of the $F_{0,audio}$, CPP_{audio}, HNR_{audio}, $Shim_{audio}$, and $Jitt_{audio}$ for saline solution (S) and the synthetic mucus samples (M1–M4).

		$F_{0,audio}$ (Hz)	CPP_{audio} (dB)	HNR_{audio} (dB)	$Shim_{audio}$ (%)	$Jitt_{audio}$ (%)
S	Median	89	22.9	7.3	4.4	3.2
(N = 893)	Mean	90 ± 15	23.4 ± 4.8	7.4 ± 3.6	5.1 ± 2.6	4.2 ± 3.4
M1	Median	92	20.8	6.8	5.5	3.0
(N = 191)	Mean	94 ± 14	21.5 ± 3.7	6.7 ± 3.2	6.5 ± 3.5	4.1 ± 3.0
M2	Median	87	22.5	8.1	4.1	3.5
(N = 202)	Mean	87 ± 16	22.5 ± 3.8	8.0 ± 3.8	4.9 ± 2.6	4.6 ± 3.6
M3	Median	90	22.4	5.6	5.4	4.9
(N = 219)	Mean	88 ± 13	22.5 ± 4.0	5.9 ± 4.0	6.2 ± 3.1	5.5 ± 4.3
M4	Median	81	21.5	6.1	5.7	3.6
(N = 155)	Mean	84 ± 16	21.5 ± 3.5	6.1 ± 3.9	6.7 ± 3.1	4.3 ± 3.2

TABLE VII. Statistical analysis of the impact of saline solution (S) and the synthetic mucus samples (M1–M4) on: (a) glottal dynamic parameters, (b) subglottal pressure parameters, (c) acoustic parameters. The p-values of statistically significant differences in the Kruskal–Wallis and Mann–Whitney U tests are printed in bold.

	Post hoc test: Mann–Whitney U ($p < 0.005$)										Kruskal–Wallis
	SM1	SM2	SM3	SM4	M1M2	M1M3	M1M4	M2M3	M2M4	M3M4	
(a) Glottal dynamic parameters											
$F_{0,HSI}$	**0.000**	0.080	0.492	**0.000**	**0.000**	**0.000**	**0.000**	0.372	0.036	**0.000**	**0.000**
GGI	0.148	0.025	0.377	0.824	**0.002**	0.867	0.134	**0.003**	0.128	0.299	**0.028**
OQ	—	—	—	—	—	—	—	—	—	—	0.187
CQ	0.802	0.362	0.180	**0.001**	0.496	0.215	0.015	0.851	**0.000**	**0.000**	**0.002**
AP	**0.000**	0.080	**0.000**	**0.000**	**0.000**	0.018	0.201	**0.000**	**0.000**	**0.002**	**0.000**
TP	**0.000**	0.374	0.006	**0.000**	**0.001**	0.035	0.187	0.146	**0.000**	**0.001**	**0.000**
PAI	0.724	0.092	0.119	0.005	0.127	0.402	0.048	0.024	**0.000**	0.359	**0.006**
ASI	0.540	**0.000**	0.062	**0.003**	**0.000**	0.291	0.040	**0.000**	**0.000**	0.269	**0.000**
(b) Subglottal pressure parameters											
$F_{0,Psub}$	**0.000**	0.081	0.485	**0.000**	**0.000**	**0.000**	**0.000**	0.373	0.032	**0.000**	**0.000**
HNR_{Psub}	**0.000**	0.261	**0.000**	**0.000**	**0.000**	0.041	0.176	**0.000**	**0.000**	0.638	**0.000**
CPP_{Psub}	0.071	0.008	0.030	**0.000**	0.485	0.806	0.011	0.583	0.055	0.013	**0.000**
$Shim_{Psub}$	**0.000**	0.703	0.014	**0.000**	**0.000**	0.022	0.801	0.018	**0.000**	0.034	**0.000**
$Jitt_{Psub}$	**0.000**	0.307	**0.000**	**0.000**	0.006	0.985	0.861	**0.003**	**0.001**	0.710	**0.000**
(c) Acoustic parameters											
$F_{0,audio}$	**0.000**	0.081	0.498	**0.000**	**0.000**	**0.000**	**0.000**	0.356	0.031	**0.000**	**0.000**
HNR_{audio}	0.032	0.011	**0.000**	**0.000**	**0.000**	0.017	0.130	**0.000**	**0.000**	0.611	**0.000**
CPP_{audio}	**0.000**	0.053	0.042	**0.000**	**0.003**	0.010	0.704	0.974	0.015	0.018	**0.000**
$Shim_{audio}$	**0.000**	0.209	**0.000**	**0.000**	**0.000**	0.417	0.371	**0.000**	**0.000**	0.087	**0.000**
$Jitt_{audio}$	0.859	0.299	**0.000**	0.667	0.467	0.006	0.780	0.050	0.647	0.022	**0.008**

The subglottal pressure parameters revealed statistically significant differences between M2 and M3 of HNR_{Psub} and Jit_{Psub} [Table VII(b)]. HNR_{Psub} decreased and Jit_{Psub} and $Shim_{Psub}$ increased with mucins (Table V).

The acoustic parameters, visualized in Fig. 5, showed similar CPP_{audio}, a decrease in HNR_{audio} and increase in $Jitt_{audio}$ and $Shim_{audio}$ with mucins. This was emphasized by the mean and median values in Table VI. Differences were statistically confirmed for HNR_{audio} and $Shim_{audio}$, [Table VII(c)], which was the same as for the subglottal pressure parameters.

IV. DISCUSSION

A. Synthetic mucus creation and expansion of the experimental setup

The viscoelasticity of human laryngeal mucus from the vocal folds determined by Peters et al.[23] could be replicated successfully by aqueous xanthan solutions of different concentrations. By adjusting the xanthan concentration, the rheology of synthetic mucus could be changed from predominantly liquid-like to predominantly elastic-like behavior. As depicted in Fig. 2, xanthan concentrations of 0.1% (M1), 0.25% (M2), and 0.75% (M4) imitated the diversity of human laryngeal mucus from the vocal folds, subdivided into Group a, Group b, and Group c.

The comparison of the absolute G' and G'' between M1 and M4 display the maximum range of viscoelasticity of the created synthetic mucus samples. The storage modulus (G'), which represents the elastic portion of the synthetic mucus sample, revealed a factor of 284 between M1 and M4 at the lower evaluation limit and 180 at the higher evaluation limit. The loss modulus (G''), which represents the viscous portion, showed a factor of 119 at the lower and 32 at the higher evaluation limit. Overall, the synthetic mucus samples covered a large frequency-dependent rheological range with viscosities and elasticities over more than 2 orders of magnitude which is similar to the range of viscoelasticities of the mean curves found for human laryngeal mucus by Peters et al.[23]

Xanthan was already used in a study by Ayache et al.[21] as mucus substitute to investigate the impact of viscosity on the vibratory characteristics of the vocal folds. However, only the viscosity, not the elasticity of the xanthan solution, was considered and the synthetic mucus was not based on data that refers to the natural viscoelasticity of human laryngeal mucus.

In addition to research regarding the impact of mucus viscoelasticity on phonation, the influence of mucins was also investigated in this study. In natural mucus, mucins are responsible for the gelling and viscoelasticity.[5] Furthermore, mucins have advantageous lubrication and hydration features.[55] However, commercially available mucins typically lost their ability to gel.[27] For this reason, 0.5% of mucin was added to 0.25% xanthan (M3) to investigate their impact. As shown in Fig. 2, the mucins did not have a distinct effect on the viscoelasticity of the xanthan solution.

The experimental setup for *ex vivo* experiments, introduced by Birk *et al.*,[24] was expanded with a method of mucus application by a modified paint spray gun. By means of fluorescence, the method was validated. The fluorescent vocal folds and the fluorescent inner tissue of the larynx [Figs. 1(c) and 1(d)] confirmed the bioadhesion of the synthetic mucus after spraying as well as after oscillation of the vocal folds. The impact of spraying on the viscoelasticity of the synthetic mucus was investigated by PTM and revealed no distinct alteration of the viscoelasticity of synthetic mucus after spraying. These results confirmed the suitability of the synthetic mucus and the application technique for the experiments.

B. General phonation parameters

The automated setup developed by Birk *et al.*[24] was expanded for mucus application. Previous studies using the same setup[36] with porcine larynges reported similar general phonation parameters as those shown in Fig. 3 and Table III. Among the 24 larynges, the parameters were homogenously distributed and gave no indication of systematic errors in the preparation of the larynges or the experimental procedure. We further assume that the interindividuality of the larynges can be neglected. A slightly higher fundamental oscillation frequency was reported with the same setup by Semmler *et al.*[36] and Birk *et al.*[35] However, the standard deviation of the fundamental frequency was about two times higher by Birk *et al.*[35] and about three times higher by Semmler *et al.*[36] Additionally only nine larynges were used in the past studies. An oscillation frequency of more than double was reported before by Alipour *et al.*[56] for porcine larynges. This discrepancy was already discussed by Birk *et al.* and might be related to the different vocal fold adduction or elongation techniques, but cannot be further explained due to a lack of provided information.[35] The predominantly linear behavior between $\bar{Q}$ and $\bar{P}_{sub}$ is in accordance with previous studies[35,36,53] as well as the logarithmic trend between SPL and $\bar{P}_{sub}$.[36,42]

C. Impact of mucus viscoelasticity on glottal dynamic parameters, acoustic, and subglottal pressure parameters

The creation of synthetic mucus for *ex vivo* experiments with realistic rheology enabled investigations about the influence of mucus viscoelasticity on phonation.

Multiple studies have investigated the impact of hydration of the larynx on phonation[15–18] but research about the impact of mucus composition and rheology is rare. In a previous study by Ayache *et al.*,[21] xanthan was used as synthetic mucus and a decrease in oscillation frequency was found with increasing viscosity. The same was found in the present study. The fundamental oscillation frequency $F_{0,HSI}$ decreased with increasing xanthan concentration, which is related to an increase in the viscoelastic moduli (G', G'') of the synthetic mucus samples, from M1 (0.1% xanthan) to M2 (0.25% xanthan) and to M4 (0.75% xanthan). However,

it increased from S to M1 (Table IV). It may be possible that the addition of xanthan at low concentration of 0.10% (M1) enhances lubrication in comparison to saline solution and leads to an increased oscillation frequency. In contrast, at higher xanthan concentrations (M2, M4), the effect of a higher elasticity and viscosity may overpower the effect of better lubrication by xanthan, resulting in a decreased frequency. The oscillation frequency also increased from M2 to M3 (0.25% xanthan, 0.5% mucin) (Table IV). This comparison reveals the impact of mucins. A better lubrication caused by the mucins[55] leading to a higher oscillation frequency may be the case. However, these assumptions must be proven in future research. An effect of synthetic mucus composition on the oscillation frequency of the vocal folds was also found by Döllinger *et al.*[4] with application of polymer solutions. Previous and present research suggest that viscoelasticity and mucus composition have an impact on the fundamental oscillation frequency of the vocal folds. The oscillation frequency may decrease with increased thickness or viscosity and elasticity of the mucus.

Similar trends were not found for the glottal dynamic parameters. Measures of glottal closure (*GGI*, *CQ*), periodicity (*AP*, *TP*), and symmetry of the left and right vocal fold (*PAI*, *ASI*), revealed no clear trends from S to M4 (Fig. 4). This is underscored by the mean and median values, given in Table IV, which indicated only small differences between the applied synthetic mucus samples. Ayache *et al.*[21] found an increased contact phase of the vocal folds for increased viscosity of xanthan solutions. It was assumed that this was due to an enhanced adhesion of the vocal folds. In this context, the *OQ* was evaluated in the present study, which gives information about the open and closed state of the vocal folds. The smaller the *OQ*, the longer the glottis is closed, which might be due to higher adhesion forces of the vocal folds. The mean values of the *OQ* decreased slightly from M1 to M4, which would be in accordance with the findings of Ayache *et al.*[21] However, an increase was also found for S to M1. Additionally, the *OQ* was the only parameter that showed no statistically significant group differences in the Kruskal–Wallis test. The absolute differences of the *OQ* were very small (Table IV). Nakagawa *et al.*[20] found that the *OQ* increased with increasing viscosity, which contradicts the findings of Ayache *et al.*[21]

To the best of our knowledge, this is the first study that systematically investigated the impact of synthetic mucus on acoustic and subglottal pressure parameters. Variation of the viscoelasticity of the synthetic mucus samples did not affect the investigated parameters and no trends were found with increasing rigidity of the mucus samples. The differences of the averaged parameters are very small among saline solution and the synthetic samples. We assume that the rheological differences of the synthetic mucus samples in the physiological range were too small to affect acoustic and subglottal pressure parameters. The investigations of mucus rheology by Peters *et al.*,[23] which provided the basis for the creation of synthetic mucus, considered only mucus of subjects without voice impairment. The diversity of

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viscoelasticity in that study and the results herein, lead to the assumption that a certain range of viscoelasticity might be tolerated by the vocal folds without an obvious effect on phonation. For future research, the rheology of mucus from subjects with dysphonia and other pathologies affecting mucus consistency should be investigated, followed by adaption of synthetic mucus viscoelasticity and conduct of experiments about the impact on phonation.

The *post hoc* Mann–Whitney U tests of the glottal dynamic, acoustic, and subglottal pressure parameters found most of the statistically significant differences between saline solution and the synthetic mucus samples between S and M4, which was the highest xanthan concentration. This comparison would lead to the assumption that the impact of mucus viscoelasticity on phonation is the highest with the highest rheological discrepancy. However, it must be considered that the absolute differences of the parameters were small and the synthetic mucus sample with the lowest xanthan concentration (M1) revealed the second highest number of statistically significant differences to saline solution for glottal dynamic parameters and subglottal pressure parameters. Moreover, within the comparison of the synthetic mucus samples, the most statistically significant differences were not found for comparison of M1 and M4, which would have supported the assumption.

The direct comparison between M2 and M3 synthetic mucus samples revealed the impact of mucins. The fundamental oscillation frequency increased slightly with mucins, which may be associated with a better lubrication of the oscillating vocal folds. The differences of the glottal dynamic parameters (Table IV) were very small and statistically significant for the GGI, AP, and ASI. For the acoustic parameters, HNR_{audio} decreased with mucins whereas $Jitt_{audio}$ and $Shim_{audio}$ increased. Hence, these acoustic parameters worsened with mucins. However, only the differences of HNR_{audio} and $Shim_{audio}$ were statistically significant. The same trends were found for the subglottal pressure parameters.

In sum, despite the large rheological range of G' and G'' over more than 2 orders of magnitude, the impact of the synthetic mucus samples and viscoelasticity on the phonatory characteristics was limited.

D. Limitations

The accessibility of the human larynx is very limited for *in vivo* measurements. Instead, *ex vivo* experiments with excised larynges enable reproducible and controllable investigations of phonation. These experiments are a common strategy in voice research in addition to computational methods[57] and synthetic vocal fold models.[58] In addition to studies with excised human larynges,[4,59] studies with larynges of animals, such as rabbits,[60] sheep,[61] or porcines[35,36] have also been used in previous studies of voice research. In general, the use of excised animal larynges in *ex vivo* studies is confronted with several commonly known limitations. In context with the present study, these are: (1) The use of

porcine larynges, instead of human cadaver larynges, limits the direct transferability of the measurement results to human phonation and the comparison to clinical data. Nevertheless, the excellent availability of the porcine larynges from a slaughterhouse enables automated studies with larynges of comparable age and physiology. (2) For high-speed recordings of the vibrating vocal folds, the vocal tract has to be removed from the larynges. This reduces the realistic boundary conditions of the experiments but enables an unhindered view on the vocal folds and characterization of the vocal fold oscillations. (3) Cartilage motions for vocal fold adduction or elongation that are caused naturally by muscles have to be integrated mechanically. Hence, the possibilities of manipulation of the cartilages of the larynx are limited.

The synthetic mucus in this study was based on xanthan, and was created with the focus on viscoelastic properties that were similar to natural human laryngeal mucus. Although the composition of mucus was not realistic, this isolation of a contributing component in a multi-factorial problem contributed to a better understanding about the impact of mucus viscoelasticity on phonation. The addition of commercially available mucins should represent a more realistic mucus.

This study only dealt with type 1 signals of regular and periodic oscillations.[54] Hence, no conclusion can be drawn on the influence of mucus on irregular or aperiodic vocal fold oscillations, which are predominantly associated with voice problems. This will be considered in future work.

For a deeper understanding of the function of mucus, further research is needed on the influence of mucus on the swelling of the vocal folds, the tissue properties, i.e., the mucosa, and the adhesion of tissue during oscillation. However, to address these research questions, living tissue and *in vivo* experiments are required due to the intact metabolism. *Ex vivo* experiments with dead tissue are not suitable.

Despite the limitations of this study, the creation of a synthetic mucus with physiological viscoelastic properties and the application on porcine cadaver larynges in *ex vivo* experiments contributed to a deeper understanding of the role of mucus in the phonatory process.

E. Conclusion

This study contributes to more realistic investigations of the phonatory process, especially regarding the role of mucus. The central achievements of this study are:

(1) Creation of synthetic mucus samples based on xanthan that are suitable for *ex vivo* experiments: Modelling of the viscoelasticity in the physiological range of human laryngeal mucus determined in a previous study by Peters *et al.*[23]

(2) Demonstration of a new application method for the synthetic mucus and verification of a sufficient coverage of mucus on the vocal folds even after oscillation experiments.

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(3) Validation of the synthetic mucus in phonation experiments: The application of synthetic mucus samples with physiological frequency-dependent viscoelasticities of a large rheological range (G', G'' over more than 2 orders of magnitude) led to phonation parameters in the physiological range with minor differences between the tested mucus groups. Thus, phonatory stability is assumed.

All in all, we increased the level of realism in this experimental replication of the phonation process as a fluid–structure–acoustic interaction by the introduction of a physiological boundary layer. This enables further systematic analysis of synthetic mucus modelled in the pathological range and its effect on the phonation parameters. An in-depth understanding of the role of laryngeal mucus will eventually lead to the development of synthetic mucus with therapeutical properties.

ACKNOWLEDGMENTS

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